UNIVERSITY OF EDINBURGH

"A study of the bacterial and fungal flora of the canine ear, with particular reference to the condition known clinically as 'Canker' (Otitis externa and media)."

Thesis presented for the Degree of Doctor of Philosophy

by

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October, 1957

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INTRODUCTION

The term 'canker' which implies ulceration with discharge has been used to describe abnormalities of the canine ear varying from a simple inflammation, or otitis, to chronic exudative otorrhoea.

Of 8,433 dogs passing through the Small Animal Clinic of this School, in the twelve months' period from July, 1953, 713 (8.4 per cent) were suffering from otitis. (Gregor, 1955). The importance of the condition lies, not only in the number of dogs affected, but in the fact that many of those cases are resistant to treatment.

The aetiology of otitis externa may be obvious, i.e. foreign bodies or ectoparasitic infestation, where removal of the offending agent is sufficient to bring about immediate relief of the clinical condition. Unfortunately, however, in the great majority of cases the aetiology is unknown and many varying methods of treatment have been employed with little success.

Cases of chronic otitis may quickly develop from the acute untreated forms as these appear to become infected with a variety of bacteria which, although not necessarily "primary pathogens", are nevertheless apparently responsible for an extension of the condition.

The importance of bacteria in affected ears has not been

been underestimated and, within recent years, many antibiotics and chemotherapeutic substances have been used to combat infection. Although most of the bacteria present are found to be sensitive, *in vitro*, to at least one of the commoner antibiotics, these more modern methods of treatment have not revealed an apparent increase in the number of cases of complete clinical recovery.

The relevant literature shows that, while the clinical aspects of otitis in dogs would appear to have been considered in some detail, there are, on the other hand, very few references to infection and the nature of the causal agents. This, in itself suggested the need for a comprehensive investigation of the bacterial and fungal flora of the healthy and infected canine ear; and is the basis of this thesis.

A REVIEW OF THE RELEVANT LITERATURE

In this thesis, the term 'canker' will be used to describe any eczematous or dermatitic lesion of the external ear, irrespective of the apparent causal agent, and will include otitis externa and media.

Otitis externa and media in man:

Predisposing factors:

In man, predisposing or exciting agents are said to be dust, or dirt in the ear, high temperature, high humidity, bathing in fresh or salt water, a low plane of nutrition, lack of personal hygiene, scalp conditions, the presence of fungi and an allergic state. Collins (1943) suggested that not one, but several of these factors may be responsible, as any circumstance which favours the growth of organisms and maceration of the skin may give rise to the condition.

Birrell (1945) observed that otitis affected all ages and social planes, and that the more important predisposing factors were vitamin deficiencies, seborrhoeic dermatitis (especially of the scalp), insects, and dirt carried by the finger nails. He also stated that irritants which enter the meatus from the middle ear, natural secretions of the meatus, external irritants, local or general allergy and skin infections will cause congestion of the meatus which may give rise to irritation and cause the patient to scratch. This, in his opinion, leads to

to a break in the surface of the skin through which saprophytic or other pyogenic organisms can gain entry. Birrell's suggestion that otitis externa results from infection, by contaminated finger nails in the process of scratching, was supported by Stewart (1951).

The main cause of the lesion in man is, according to Brown (1937), the invading organism or 'virus' which reaches the epithelium of the external ear either by the blood stream or from an external source.

Glandular and nutritional disorders, including vitamin deficiencies, may play a part in lowering the patient's resistance to the invading micro-organism and, in this connection, the work of Turner and Loew (1931) is of interest. While studying sinus infections in rats which were on a vitamin A deficient diet, they found a marked difference between the bacterial flora of normal and avitaminosis-A animals, whether or not suppuration was present in the sites examined. The increase in the numbers of different organisms which appeared in the avitaminosis-A rats, as compared with the controls, suggested that lack of this vitamin may render an animal more prone to infection.

Seborrhoeic dermatitis (Ingram, 1939), eczema and scalp conditions have also been mentioned as predisposing factors to external otitis in man. This may also be the case with dogs many of which suffer from dermatitis, pruritus, eczema, labial dermatitis, anal adenitis, and skin conditions due to

to ectoparasitic infestation.

The bacterial and fungal flora of the human ear:

There are numerous reports on the bacterial and fungal flora of the infected human ear, the results of which are in fairly wide agreement, but there is still much speculation as to the aetiological significance of the various micro-organisms.

In Brown's (1937) opinion the probable cause of otitis, in man, is the invading organism or 'virus', e.g. staphylococci, Streptococcus longus and the 'acne bacillus' (Pityrosporum). Although these organisms may cause the commoner type of eruption, other clinical forms of otitis may be due to species of Aspergillus, Pseudomonas, and Corynebacterium.

Birrell (1945) found that otitis externa accounted for 25 per cent of aural cases in British Service personnel. Bacteriological examination usually resulted in the isolation of Ps. pyocyanea although in some cases 'diphtheroids', Pr. vulgaris, and Staph. albus were recovered.

In their survey of 200 cases of otitis in man Friedman and Hinkel (1941) reported that, in every instance, more than one organism was present of which Staph. albus, 'diphtheroids', Ps. pyocyanea and micrococci, were the commonest. Similar results were obtained by Daggett (1942).

In America, Salvin and Lewis (1946) studied 100 consecutive cases of otitis in Naval personnel and found that bacteria only were present in 84, fungi only in 8, and bacteria and fungi

fungi together in the remaining 8 cases. Of the 16 fungi there were 11 Aspergillus species, 1 Monotosporon species and 4 strains of Actinomyces israeli (sic.). Of the ears from which bacteria were recovered, 45 had Pseudomonas, 27 Staph. albus, 14 'diphtheroids', 9 Streptococcus viridans, 2 haemolytic streptococci, 1 Staph. aureus, 9 Chromobacterium species and 2 Sarcina lutea. Of the 25 healthy ears 17 showed Gram-negative rods, 16 Staph. albus, and 4 were apparently sterile. Salvin and Lewis also referred to the work of Minilew, Collins and Harris (1940) who claimed that fungi, especially Aspergillus species, are important causal organisms of otitis. Although this opinion was shared by Gill (1938), Whalen (1938) and Dobie (1943) other workers including Williams, Montgomery and Powell (1939) have shown that fungi were present in a minority of cases and that bacteria are the commonest organisms in diseased ear canals.

Stewart (1951) described the bacteriology of 126 cases of chronic exudative otitis, as well as that of a number of healthy ears, in man. Thirty-four of 104 healthy ears were said to be sterile although Staph. albus and Staph. aureus were isolated from 55 and 5 ears, respectively. His findings from the otitis cases were quite different, as species of Proteus, Pseudomonas, staphylococci, Escherichia and 'enterococci' occurred alone, or together with other organisms, in 94 out of 126 cases.

In his survey of ear, nose and throat casualties in the Middle East, Collins (1943) reported that the percentage of

of cases of otitis externa was approximately equal to that of otitis media. He also found a higher incidence of Staph. aureus, than did Daggett (1942) and that, although streptococci were rarely isolated, 'diphtheroids', Proteus and Pseudomonas were often present.

Dodge (1935) mentioned several reports of the isolation of fungi from the ear of man. These included 9 species of Aspergillus, 2 species of Absidia, and species of Mucor, Bargellinia, Hemispora, Myceloblastonon, Actinomyces, Discomyces, Penicillium and Rhinosporium.

Ainsworth (1952) noted the isolation of Absidia corymbifera from the ear of man, while other species of fungi have been described by Martin (1936) and Lacroix, Riser and Karlson (1947). In addition, an unknown visitor at the 1951 Meeting of the Royal Society of Medicine stated that 39 fungal species had been reported from healthy and infected human ears.

In his survey of fungal diseases in Great Britain, Duncan (1949) mentioned 3 cases of Candida albicans from acute mycotic otitis in man, which were apparently secondary to a staphylococcal otitis media; and two cases of Otomycosis (Tropical Ear) due to Aspergillus flavus.

According to Lumsden (1951), however, the most common cause of external otitis, in man, is the yeast Pityrosporum.

Otitis externa and media in the dog:

Predisposing factors:

The predisposing factors of canine otitis were summarised by Kirk (1948) who considered that the presence of dirt, wax, foreign bodies, excessive moisture, growths or mechanical interference favours the onset of the disease. Kral (1952) extended this list to include fungi and ear mites, as well as a number of generalised infections such as distemper, the complications of which may give rise to pustular lesions in the external acoustic meatus.

Many authors have stressed the part played by ectoparasitic infestations in the aetiology of canine otitis, the single species of the genus Otodectes, O. cyanotis, being parasitic on the dog, cat, fox and ferret. This mite attacks the interior of the external auditory meatus (although on occasions, it may spread beyond the ears) giving rise to inflammation of the lining membrane with exudation of lymph and the formation of crusts in the ear canal. (Lepage, 1956).

Kaufman and Frost (1949) are also of the opinion that otitis is frequently caused by ear mites as 56 per cent of dogs were shown to be infested while being weaned. Woodrow (1953) refers to other findings by the same authors when they observed ectoparasites in 90 per cent of cases.

Jennings (1953) has shown that mites may be few in number in cases of otodectic otitis in the dog, although the presence of

of even an occasional parasite can cause a great deal of irritation. He concluded that most dogs are infected by cats, which are commonly infested with large numbers of parasites, and that, where a dog is being treated for otodectic otitis, it is important that any cat in the same household be also treated.

Although Holmes (1933) and Pugh (1947) emphasised the part played by ear mites, Georgi (1952) has shown that Otodectes species are seldom found in cases of canine otitis. Beresford-Jones (1955) found, after a three years' survey of the incidence of O. cyanotis in dogs and cats in London, that between 2.5 and 3.5 per cent of dogs, and between 20 and 28 per cent of cats harboured this parasite.

Kral (1952), McGinnis and England (1949) and Kaplan (1951) stressed the importance of long ears as a predisposing factor. The first named found otitis to be commoner in long eared, long haired breeds such as the spaniel and Irish setter, while Georgi (1952) reported that of 100 dogs with otitis, the ears of 70 were drooping, 20 were erect, and 10 were cropped.

The bacterial and fungal flora of the canine ear:

There is little reference in the literature to the flora of the normal healthy canine ear and very little work appears to have been published on the bacteriology of the cankerous ear, as most reports are of a clinical nature.

Dam (1952) studied the bacterial flora of canine otitis externa and carried out antibiotic sensitivity tests on the

the organisms isolated. Of the 17 cases examined, 16 showed a Staphylococcus in pure culture and the other case a mixed infection of staphylococci and Pseudomonas pyocyanea.

Farrag and Hosny Mahmoud (1953) drew attention to the high incidence of otorrhoea in dogs due to Ps. aeruginosa and showed that the condition could be reproduced within ten days by instilling saline suspensions of Pseudomonas cultures into the external ear canals of healthy dogs. Although McGinnis and England (1949) stated that fungi and bacteria may irritate the aural lining, they did not name the organisms to which they referred; but in the summary of their paper they suggested that ear mites are probably the initial cause of otitis in dogs.

Serth (1954) submitted material from 27 cases of canine otitis for bacteriological examination and reported the results obtained. Staphylococci were recovered from 14 cases, Proteus vulgaris from 3 cases and Ps. aeruginosa from 2 cases. The remaining 6 cases were described as 'negative'.

Of greater interest are the preliminary reports of Gustafson (1954) and Jones (1955). Gustafson carried out a number of experiments in which he induced otitis by injecting organisms isolated from otitic material into the external acoustic meatus of healthy dogs. The bacteria present in infected ears occurred in the following order of frequency:- staphylococci, yeasts, Gram-positive bacilli, haemolytic streptococci, Gram-negative bacilli, Pr. vulgaris, Ps. aeruginosa

Ps. aeruginosa and coliform organisms. Jones (1955) found that the predominant organism in canine otitis was Staph. aureus and that other bacterial species occurred in the following order of frequency:- Pr. vulgaris, Ps. aeruginosa, Bacillus subtilis, 'diphtheroids', non haemolytic streptococci and Escherichia coli.

Apart from the ringworm group, which has received the attention of numerous workers in the past, there are occasional references in the literature to other fungal species from the skin of the dog which are of interest to this work.

Meyer (1912) described ulcerative blastomycosis in the head of a dog, Madsen (1942) reported on Blastomyces dermatitidis from animals and Foshay and Madden (1942) discussed the dog as a natural host of this fungus. In his preliminary report, Schoop (1951) described 'blastomycetes' in infected ears and although they were also present in the ears of healthy dogs, he nevertheless considered them to be aetiologically important. The same opinion was expressed by Gustafson (1954) who described the organism as Pityrosporum species, a yeast which has also been isolated from an eye lesion in a dog. (Ainsworth, 1954).

This review of the available literature shows that, in comparison with the amount of work published on human otitis, there are but few references to the bacteriology of otitis, or 'canker', in the dog.

As this study of the bacterial and fungal flora of the canine ear refers particularly to the condition of otitis, it will be convenient to discuss the findings under the following headings:-

Part I. The bacterial and fungal flora of the ears, tonsils and anterior nares of clinically normal dogs.

Part II. The bacterial and fungal flora of the ears of dogs suffering from otitis, swabs being taken before the commencement of treatment: with a comparison of the findings in Parts I and II.

Part III. The bacterial and fungal flora of dogs suffering from otitis which failed to respond to treatment.

Part IV. The factors predisposing to otitis.

Part V. The histopathology of the external ears of dogs suffering from otitis.

Part VI. A detailed study of the micro-organisms isolated from the canine ear.

Part VII. The sensitivities to antibiotics of the more commonly occurring bacteria in external otitis.

MATERIALS AND METHODS

The external and middle ears, the anterior nares and the tonsils were swabbed and the material inoculated on glucose broth, nutrient broth, a horse blood agar plate and two maltose or malt agar plates. In addition, cooked meat medium was inoculated with material from the outer and middle ears. Rectal swabs were inoculated on to one each of glucose broth, nutrient broth, Selenite F broth, and plates of horse blood agar, nutrient agar, and MacConkey's bile salt medium.

All the inoculated media were incubated at 37°C. for 24 hours or longer, except for nutrient broth and half of the nutrient agar, maltose and malt agar plates, which were kept for 2 to 3 weeks at 25°C. The cooked meat media were incubated for 48 hours at 37°C., when they were streaked on horse blood agar plates which were incubated anaerobically for two days, at 37°C.

Pure cultures were obtained of the different organisms which were examined, provisionally identified and retained in stock culture, on nutrient agar slopes or cooked meat medium.

Media:

Most of the media used routinely in this investigation were prepared according to the methods of Mackie and McCartney (1949). These were:- glucose broth, nutrient broth, peptone water, cooked meat medium, Selenite F broth, horse blood agar, MacConkey's bile salt medium, nutrient gelatin, Loeffler's serum

serum medium, litmus milk and methylene-blue milk media and liquid fermentation media ('sugars'). Other media used were as follows:- Sabouraud's maltose (and glucose) agar, (Ainsworth, 1953), malt agar (Lodder and van Rij, 1952), Raulin's medium, (Smith, 1947), corn-meal agar (Lodder and van Rij, 1952), fermentation media for yeasts, (Lodder and van Rij, 1952), milk agar, (Christie and Keogh, 1942), and nitrogen-free fermentation media, (Liu, 1952).

The preparation of the media and the methods used to study various reactions were, unless otherwise stated in the text, in accordance with the recommendations of the following authors:- Nitrate reduction,(Kauffman, 1954), Voges-Proskauer and Methyl Red tests, (Kauffman, 1954), ammonia from peptone water, (Shaw, Stitt and Cowan, 1951), Indole from peptone water, (Mackie and McCartney, 1949), catalase production, (Isaacs and Scouller, 1948), hydrolysis of sodium hippurate, (Little and Plastridge, 1948), hydrolysis of aesculin, (Little and Plastridge, 1948), decomposition of urea, (Christensen, 1946), citrate utilisation, (Mackie and McCartney, 1949) and hydrogen sulphide production, (Kauffman, 1954).

The staining methods and the preparation of the following stains and reagents were all according to Mackie and McCartney, (1949); except where stated:- Loeffler's alkaline methylene blue, Jensen's modification of Gram's staining method, Ziehl-Neelsen method of staining acid-fast bacilli, Neisser's method (modified)

(modified) of staining volutin granules, Muir's method of staining capsules, negative staining methods, haematoxylin and eosin staining method, Gomori's aldehyde fuchsin method of staining, (Gomori, 1950), Periodic acid-Schiff stain - the Hotchkiss-McManus technique, (Kligman and Nescon, 1950), and Lacto-phenol cotton blue, (Ainsworth, 1953).

PART I.

The bacterial and fungal flora of the external and middle ears, the anterior nares and the tonsils of clinically healthy dogs

It is desirable that the flora of the ears of healthy dogs be considered before discussing the significance of the micro-organisms in infected ears.

In the clinically "normal" animal, material was obtained from the proximal part of the external acoustic meatus at the point where the annular cartilage turns through an angle of almost 90° before it reaches the tympanic membrane.

The first ten dogs in this series were exceptional, however, in that additional swabs were taken from the region of the external opening to the auditory canal and also from the inner surface of the ear flap.

In man, middle ear infections are very often secondary to septic foci in the tonsils or the naso-pharyngeal region while in dogs, although clinical otitis media is rarely diagnosed, it is also possible for pathogenic bacteria in the throat to gain access to the middle ears by way of the eustachian tubes. This middle ear infection may, in turn, extend to the external acoustic meatus giving rise to a typical otitis externa, with or without perforation of the tympanum.

With these points in mind, it was decided to extend the examinations of healthy dogs to include material from the anterior nares and tonsils.

A number of rectal swabs from the same dogs were also examined in order to study the characteristics of potentially pathogenic faecal strains; the intestine being a possible reservoir of infection.

The sampling of the middle ears presented a problem which was only overcome by selecting fresh cadavers (the dogs having been destroyed at the owners' request for such reasons as "biting", "moving house", etc.), every effort being made to ensure that the dog was clinically healthy at the time of death and that the ear canals were entirely normal. In each case the autopsy was completed within an hour of death and the necessary cultures made within two hours of the animal being destroyed. Access to the middle ear was obtained by removing the tongue and the soft tissues in the region of the naso-pharynx to expose the osseous bulla. The outer bony casing was gently flamed and reflected to expose the intact lining membrane, which was examined for evidence of congestion, or other abnormality, before being carefully removed with sterile instruments and the swabs inserted into the cavity of the middle ear. The external auditory meatus was then examined for evidence of discharge, ulceration, congestion and foreign bodies.

After taking the necessary samples, an inch long section of the cartilagenous meatus was removed and fixed in 10% Formol saline.

If the histological preparations showed thickening of the

the stratified squamous epithelium, inflammatory reaction, or alteration in the appearance or distribution of the glandular structure, all cultures were discarded and the case was rejected as being "not normal".

In addition each swab was smeared, stained and examined microscopically.

Material:

Including the first ten dogs, the ears of which were examined at three different levels, 35 healthy dogs were accepted as being "normal". The number of sites examined was as follows:-

External Ears	70
Middle Ears	50
Anterior nares	35
Tonsils	35
Rectal Swabs	30

Age: Sex and Breed:

The healthy dogs were chosen at random, no attempt being made to choose the animals by age, sex or breed. Twenty-three were dogs, twelve were bitches and the average age was 3 years and 9 months. In most cases, the breed of dog was obvious but in four cases, the characteristics were not well defined and it was necessary to describe them as "cross terriers".

TABLE 1.

The Breed and Sex of the 35 Healthy Dogs

Breed	Male	Female	Total	Breed	Male	Female	Total
Spaniel	5	2	7	Greyhound		1	1
Collie	4	2	6	Elkhound	1		1
Labrador	2	2	4	Poodle		1	1
"Cross Terrier"	4		4	French Bulldog		1	1
Alsatian	1	2	3	Cairn	1		1
Scottie	1	1	2	Boxer	1		1
Retriever	1		1	Lurcher	1		1
Dachshund	1		1	Total	23	12	35

In addition to the details mentioned above, 18 dogs had "Prick" or "semi-prick" ears and 17 dogs had "drop ears".

As analysis of the findings from the first ten dogs (Appendix 1) suggested that the continued sampling of three different sites of the same ear would be superfluous, the examination of all other healthy ears was confined to material obtained from within the auditory canal at a point approximately one inch from the external opening.

This survey of the external and middle ears, the anterior nares and the tonsils of healthy dogs showed the presence of a large number of different bacterial species, many of which were potential pathogens.(Table 2.)

TABLE 2.

The flora of the ears, nares, and tonsils of healthy dogs

	Outer Ears		Middle Ears		Nares		Tonsils	
	No.	%	No.	%	No.	%	No.	%
Pseudomonas	-	-	-	-	-	-	-	-
Proteus	-	-	-	-	-	-	2	(6)
Coliforms	4	(6)	6	(12)	5	(14)	22	(63)
Haemolytic streptococci	2	(3)	3	(6)	4	(11)	12	(34)
Non haemolytic streptococci	21	(30)	7	(14)	16	(46)	18	(51)
Staphylococci	39	(54)	9	(18)	25	(71)	17	(49)
Diphtheroids	11	(16)	1	(2)	6	(17)	8	(23)
Subtilis group	22	(31)	3	(6)	14	(40)	8	(23)
Micrococci	12	(17)	6	(12)	9	(26)	3	(9)
Other Gram-negative rods	3	(4)	7	(14)	11	(31)	19	(54)
Miscellaneous organisms	15	(21)	10	(20)	18	(51)	25	(71)
Sporing anaerobes	31	(44)	2	(4)	NOT EXAMINED			
Pityrosporum	25	(36)	-	-	-	-	-	-
Other yeasts	3	(4)	-	-	-	-	2	(6)
Fungi	8	(11)	-	-	4	(11)	-	-

NOTE:- These figures indicate the number of times a particular organism was recovered from a given site.

These results show a number of interesting features. The commonest bacterial species was the Staphylococcus which occurred in 54 per cent of the external ears, although the degree of growth, in primary cultures, suggested however that they were never present in large numbers. Other common organisms were the anaerobic sporing bacilli which were rarely seen in smears but were recovered in small numbers from 31 (44 per cent) of the external ears. The great majority of the anaerobic species were identified as Clostridium welchii, a number of which produced type A. toxin. (See Part II). Haemolytic streptococci do not appear to be commensals of the external auditory meatus and even the non haemolytic species, which were present in 30 per cent of ears, were rarely numerous. There was no evidence of species of Proteus and Pseudomonas, organisms which are so frequently present in large numbers in the purulent or more chronic forms of otitis, such as otorrhoea.

Cultures from the middle ears were frequently sterile, although a number showed a variety of Gram-negative rods, or cocco-bacillary forms. Coagulase negative staphylococci, micrococci, and non haemolytic streptococci were not infrequent however. The tonsil swabs were of interest in that approximately every third dog was found to carry haemolytic streptococci. Species of Pasteurella were frequently encountered in the tonsils, although typical Pasteurella septica did not appear to be a normal inhabitant of these tissues. The fact that coliform

coliform organisms were present in 63 per cent of the tonsil swabs was quite unexpected, and is difficult to account for.

The flora of the anterior nares of normal dogs showed an equally unexpected finding in that the carrier rate for staphylococci was not less than 71 per cent, although, it must be added, in most cases only an occasional colony was present on primary plate cultures. Again, in a number of cases, it was only after the fluid media had been incubated for two days that there was evidence of growth from the original swabs.

The presence of Pityrosporum species in cultures prepared from 36 per cent of ears is noted, and will be referred to later.

It must be emphasised that it is not intended to include, at this stage, full details of the characteristics of each bacterial species that was isolated from normal animals, as such data would tend to overshadow the main argument. (These will be considered in Part VI). Nevertheless, in order to identify the strains sufficiently, and so provide a basis for comparison between the normal and infected tissues, the more important characteristics will be included in the discussion that follows.

Pseudomonas:

Pseudomonas aeruginosa is one of the most important species to be found in cases of purulent otitis. That the condition is serious in dogs is due, not only to the inaccessability of the deeper parts of the external auditory meatus, but also to the fact that the organism is comparatively resistant to most therapeutic substances. Extension of the infection to the middle ear is not uncommon and, in dogs, this usually results in the animal being destroyed.

It is, therefore, of interest that there was no evidence of Pseudomonas in the healthy external and middle ears. This suggests that the organism reaches the lesion in the ear either from an outside source such as water, or sewage, or as the result of auto-infection from the alimentary canal.

Proteus:

It will be shown, in Part III, that Proteus species and in particular Proteus mirabilis, play an important part in both the acute and chronic forms of otitis, although in the latter condition, it is difficult to distinguish clinically between Proteus and Pseudomonas infections.

In none of the healthy ears, tonsils or nares was there evidence of Pr. mirabilis, although Pr. vulgaris, which is a much rarer species in dogs, (Part VI.b.), was recovered in cultures of the tonsils of two normal dogs and then only after the cultures had been incubated for four days.

The rectal swabs, on the other hand, showed a surprisingly high incidence of Proteus (47%), 3 swabs giving Pr. vulgaris and 11 swabs Pr. mirabilis, of a total of 30 swabs examined. All were Gram-negative, non-sporing, motile rods which, with one exception, showed a swarming type of growth on 1.5% nutrient agar. They all fermented glucose, but not lactose, with the production of acid and visible gas. The rapid hydrolysis of urea and the reduction of nitrates was a constant feature of all strains, as was the liquefaction of gelatin and the production of hydrogen sulphide.

The Pr. vulgaris strains were identified by the presence of acid and gas in both maltose and sucrose, within twenty-four hours, and by their ability to produce indole in peptone water cultures.

The most important characteristics of the two tonsil, and fourteen rectal strains, from healthy dogs are summarised in Table 3.

These findings show that strains of Pr. mirabilis and Pr. vulgaris of canine origin conform very much to type. The production of acetylmethylcarbinol by seven of the Pr. mirabilis strains was demonstrated by the methods of Barritt (1936), none of the Pr. vulgaris strains giving a positive Voges Proskauer reaction. Detectable amounts of indole were produced in peptone water cultures by Pr. vulgaris but not by Pr. mirabilis. Pr. vulgaris was also identified by its ability to ferment maltose and frequently salicin.

TABLE 3.

The characteristics of 16 strains of Proteus from healthy dogs

Number of Strains giving a positive reaction

	Glucose	Lactose	Maltose	Mannitol	Dulcitol	Inositol	Sucrose	Trehalose	Xylose	Rhamnose	Dextrin	Arabinose	Sorbitol	Mannose	Galactose	Laevulose	Salicin
Pr. mirabilis	11	0	0	0	0	0	10	11	11	0	0	0	0	0	11	10	0
Pr. vulgaris	5	0	5	0	0	0	5	5	5	0	0	0	0	0	5	5	3

	Swarming	Indole	Motility	Urea	H_2S	Gelatin	Solidified Serum	Citrate	Nitrates	Methylene Blue	Voges Proskauer	Methyl Red
Pr. mirabilis	11	0	11	11	11	11	11	10	11	11	7	11
Pr. vulgaris	5	5	5	5	5	5	5	5	5	5	0	5

The Coliform Group:

The term "coliform group" will be used in accordance with the recommendations of Kauffman (1954) to include the genera Escherichia, Klebsiella, Gloaca and Hafnia.

Members of the coliform group were present in 6, 12, 14 and 63 per cent, respectively, of the external and middle ears, the anterior nares and the tonsils of healthy dogs. In addition to the above, 25 coliforms were selected at random from the rectal strains and submitted to detailed investigation.

Escherichia coli:

The organisms in this group were identified as Gram negative, usually motile, non sporing rods which generally formed indole, were V.P. negative and M.R. positive but did not hydrolyse either urea or gelatin. Nitrates were invariably reduced to nitrites and most strains failed to grow in Koser's medium. All typical strains usually fermented at least glucose, maltose and mannitol, although a few failed to ferment lactose promptly. None of the strains attacked inositol and certain other sugars.

It is permissible, at this stage, to distinguish the various types of E. coli by the following four (I. M. Vi. C.) reactions namely, the production of indole and acetylmethylcarbinol, the methyl red test and the utilisation of sodium citrate as the sole source of carbon.

TABLE 4.

Types of E. coli from healthy dogs

Site Examined	Number of Strains Examined	Number of Strains Conforming to type	Indole	M.R.	V.P.	Citrate
External ear	3	2	+	+	-	+
		1	+	+	-	-
Middle ear	5	5	+	+	-	-
Nares	4	4	+	+	-	-
Tonsil	15	11	+	+	-	-
		1	+	-	+	-
		1	+	-	+	+
		2	+	+	-	+
Rectum	23	22	+	+	-	-
		1	+	+	-	+
	50					

The above results give an indication, not only of the different types of E. coli but also of their probable source of origin. Unfortunately, only three strains were present in the healthy external ears but of the others, all those from the middle ears and the anterior nares and most of the tonsil strains were very similar to twenty-two of the twenty-three rectal strains.

In the Reports on Public Health and Medical Subjects, No.71 (1956), coliforms giving these results are described as B. coli, type I, the majority of which are thought to be primarily of intestinal origin. A more detailed discussion of this group of organisms is included in Part VI.c. of this thesis.

Escherichia freundii:

This species differs from E. coli in that indole is not usually produced in peptone water cultures, although the reactions to the Methyl Red and Voges Proskauer tests are similar.

Only three strains of E. freundii were isolated from healthy dogs and all were from tonsil swabs. They were motile, indole negative, V.P. negative, M.R. positive, Eijkman negative and grew well in Koser's medium. Methylene blue milk and nitrates were reduced but gelatin and urea were not hydrolysed. Acid and gas were produced by the fermentation of glucose, lactose, maltose, mannitol and certain other sugars. In dulcitol and sucrose the reactions were variable whereas salicin, and a number of other sugars, were not attacked.

Klebsiella species:

Klebsiella (aerobacter) species were identified as members of the tribe Escherichieae which differed from the typical E. coli in the following important respects. They were non-motile, indole negative and M.R. negative rods which grew well in Koser's medium, produced acetylmethylcarbinol in glucose phosphate broth

broth and fermented inositol, sucrose and salicin.

Klebsiella species were isolated from three of the external ears and two of the tonsils.

In addition, to the features described above, all five strains fermented glucose, lactose, maltose, mannitol, sorbitol, raffinose, arabinose, trehalose, xylose, rhamnose, glycerol, fructose, galactose and mannose. Dulcitol and inulin were not fermented.

Nitrates and methylene blue were reduced, urea was slowly decomposed and although litmus milk showed a slight degree of acidity, the formation of a soft coagulum was variable. Gelatin was not liquefied nor was hydrogen sulphide produced.

Cloaca, Hafnia and 'Paracolon' species:

Seven strains could not be included in the previous groups as they failed to ferment lactose after three weeks' incubation.

One strain, from the tonsil, was finally identified as Cloaca species, as it closely resembled the previously designated Aerobacter cloacae. This Gram-negative, motile rod which was indole negative, M.R. negative, V.P. positive and citrate positive liquefied gelatin slowly. Acid was produced in saccharose and salicin after 48 hours although all other fermentable sugars were split promptly. There was no change in lactose, dulcitol, inositol and inulin.

Three strains were classified as Hafnia species as they failed to ferment lactose, dulcitol or inositol. Sucrose was

was fermented by one strain after 72 hours, after 14 days by a second and not at all by the third strain. One of the three strains failed to ferment salicin. These reactions, and the fermentation of glucose and mannitol, were typical of the Hafnia group, as were also the inability to produce indole and the non-liquefaction of gelatin.

The "paracolon group" is not well defined and three strains, which were recovered from one tonsil and two rectal swabs, were so variable in many of their reactions that it was not possible to include them in any of the other groups. As they also failed to ferment lactose, it may be permissible to call them "paracolon organisms".

As only a very few strains of Cloaca, Hafnia and paracolon species were isolated from normal dogs, their presence was probably of little importance.

B. faecalis alkaligenes and B. alkaligenes metalkaligenes:

The genus Alkaligenes, as defined by Bergey (1948), includes small Gram-negative rods which may or may not show motility and which do not produce acid or gas from the usual sugars. In litmus milk the reaction is usually alkaline and neither gelatin nor solidified blood serum is regularly liquefied. Acetylmethyl-carbinol is not produced by glucose phosphate broth cultures.

In normal dogs two distinct types of Alkaligenes faecalis were recovered, the first resembling the strains of Petrushky

Petrushky (1896) and the second, which is referred to in this thesis as Alkaligenes metalkaligenes , resembling Nyberg's (1935) B. alkaligenes.

The distribution of these strains in healthy dogs is shown in Tables 5 and 6.

TABLE 5.

B. alkaligenes metalkaligenes from healthy dogs.

Site examined.	Number of strains.	Motility	Indole	V.P.	Nitrates	Citrate	Fermentation of sugars.
External ears	1	-	-	-	-	+	-
Middle ears	0						
Nares	2	-	-	-	+	+	-
Tonsils	4	-	-	-	-	-	-
	1	-	-	-	+	+	-

TABLE 6.

B. alkaligenes faecalis from healthy dogs.

Site examined	Number of strains	Motility	Indole	V.P.	Nitrates	Citrate	Fermentation of sugars.
External ears	1	+	-	-	+	+	-
	1	+	-	-	+	-	-
Middle ears	1	+	-	-	+	-	-
Nares	1	+	-	-	-	-	-
Tonsils	2	+	-	-	-	-	-
	1	+	-	-	-	+	-

Staphylococci:

The staphylococci were identified as catalase positive, non-motile, non-capsulate, Gram-positive cocci, occurring in irregular masses but never in long chains. Growth was abundant on most media and many sugars were fermented with the production of acid but not gas. Both pigment production and proteolytic activity were variable.

Each strain was examined in detail but, for the present, only the following will be considered, viz:- coagulase and haemolysin production, (see Part VI.d.), the fermentation of lactose, maltose and mannitol, and the liquefaction of gelatin and solidified serum. These features will be sufficient to enable a comparison to be made of the strains which were present in the ears, nares and tonsils of healthy dogs.

The incidence of staphylococci in the external and middle ears, nose and tonsils was 54, 18, 71, and 49 per cent respectively.

In all, 133 strains were studied and by means of the coagulase test they were immediately classified as potentially pathogenic or non pathogenic staphylococci.

The figures in Table 7 show that, of the four sites examined, the anterior nares was the most important source of staphylococci, 68% of which were potential pathogens. Staphylococci were present in just over half the external ears examined and, although only 46% were coagulase positive, these figures

figures suggested that the staphylococci which are normally present in dogs' ears may play an important part in the development of the primary lesion of otitis.

TABLE 7

The incidence of potentially pathogenic staphylococci in healthy dogs

Site Examined	Number of Strains Examined	COAGULASE. Produced	Not Produced
Outer ears	76	35	41
Middle ears	12	2	10
Anterior nares	25	17	8
Tonsils	20	9	11
Total	133	63	70

TABLE 8

The characteristics of 63 strains of Coagulase Positive staphylococci from healthy dogs

Number of Strains from:-				Haemolysin	Haemolysin	Haemolysin	Gelatin Liquefied	Solid Serum Liquefied	Lactose	Maltose	Mannitol
Nose	Tonsil	Mid Ear	External ear	α.	β.	δ.					
1	-	-	1	+	+	+	+	+	+	+	+
-	-	-	2	+	+	+	+	+	+	+	0
4	1	-	5	+	0	+	+	+	+	+	+
11	2	-	2	+	0	+	+	+	+	+	0
6	2	-	6	0	+	+	+	+	+	+	+
4	3	2	16	0	+	+	+	+	+	+	0
-	-	-	2	0	+	+	+	+	+	0	0
-	-	-	1	0	+	0	+	+	+	0	+
1	-	-	-	0	+	+	+	+	0	+	0
-	1	-	-	0	+	+	+	0	+	+	0
17	9	2	35	19	48	62	63	62	62	60	27

NOTE: The methods for the detection of staphylococcal haemolysins are discussed in detail in Part VI.d.

TABLE 9

The Characteristics of 70 strains of Coagulase Negative staphylococci from healthy dogs

Number of Strains from:-				Haemolysin	Haemolysin	Haemolysin	Haemolysin or none	Gelatin Liquefied	Solid serum Liquefied	Lactose	Maltose	Mannitol
Nose	Tonsil	Mid ear	External ear	α.	β.	δ.	ε.					
-	-	-	2	0	+	+		+	+	+	0	0
-	-	-	4	0	+	0		+	+	+	+	0
-	-	-	1	0	+	0		+	+	0	0	+
-	1	-	1				+	+	+	+	+	+
-	1	-	1				+	+	+	+	+	0
-	-	-	3				+	+	+	0	+	+
-	1	-	-				+	+	+	0	+	0
-	-	-	1				+	+	+	0	0	0
4	1	-	4				+	+	0	+	+	+
-	-	-	2				+	+	0	+	+	0
-	-	1	-				+	+	0	+	0	+
-	1	1	2				+	+	0	0	+	0
1	-	3	1				+	+	0	0	0	0
3	6	5	19				+	0	0	v.	v.	v.
8	11	10	41	0	7	2	63	37	16	35	50	19

v. = variable fermentation reaction

The results in Table 8 show that most coagulase positive staphylococci produce either beta and delta haemolysins or alpha and delta haemolysins, irrespective of the source of the strains. Gelatin and solid serum were invariably liquefied and lactose and maltose were promptly attacked. The fermentation of mannitol did not appear to be a feature of pathogenic staphylococci of canine origin.

Only seven coagulase negative strains (Table 9), and all of them from external ears, produced beta haemolysin, two of which were exceptional in that they produced delta haemolysin but not coagulase. This will be referred to in Part VI.d., when the staphylococci are discussed in detail.

Unlike the coagulase positive staphylococci, there did not appear to be a predominant type amongst the 70 non pathogenic strains, which suggests that most of them are not of aetiological significance in canine otitis. Also, most coagulase positive strains fermented lactose whereas the majority of the coagulase negative strains attacked maltose. It may also be mentioned, at this stage, that potentially pathogenic dog staphylococci rarely produced the classical aureus pigment. Of the 63 coagulase positive strains from healthy dogs, only 16 (25.4 per cent) produced golden, khaki or aureus pigment on 33 per cent milk agar plates, while the corresponding figure for coagulase positive staphylococci from infected ears was only 9 per cent.

Streptococci:

These were distinguished from staphylococci as Gram-positive cocci occurring singly, in pairs or in chains but never in packets. They were all non-motile, catalase negative and nitrate negative aerobes that did not grow profusely on artificial media. All strains were broadly classified according to the type of growth on 5 per cent horse blood agar. The presence of zones of complete haemolysis about the colonies differentiated the haemolytic streptococci from the non-haemolytic strains, which had no effect on horse red cells. A number of other characteristics was studied including, in the case of all the haemolytic strains, the precipitation reactions of Lancefield (1933).

Haemolytic Streptococci:

These were present in 3, 6, 11 and 34 per cent respectively of the external and middle ear, anterior nares, and tonsil swabs of healthy dogs.

The incidence and distribution of types of haemolytic streptococci from the various sites were as follows:-

TABLE 10

Haemolytic streptococci from the ears, nares and tonsils of healthy dogs

Number of strains and the sites examined		Lancefield's Groups							
		A	B	C	D	F	G	L	M
External ears	2	-	-	1	-	-	1	-	-
Middle ears	3	-	-	-	-	-	1	-	2
Nares	6	-	-	-	-	-	3	2	1
Tonsils	18	-	1	3	-	-	9[x]	-	5
Rectum	5	-	-	-	2	-	3	-	-
Total	34	-	1	4	2	-	17	2	8

x Includes 3 strains from the same dog

The 5 rectal strains were chosen at random from the same healthy dogs and are included here for comparison.

These results show that in the normal dogs the Group 'G' strains were the commonest type of haemolytic streptococcus present, the other strains occurring in the following descending order of frequency, viz:- Groups, M, C, L, D and B. The high percentage of Group 'M' strains in the tonsils of healthy dogs was of interest as this type of streptococcus was rarely present in otitic material which suggested that the Group 'M' haemolytic streptococci were commensals of the tonsils and naso-pharynx of

of normal dogs.

On the other hand, Group 'G' strains were commonly found in healthy tissues and accounted for 50 per cent of the total number of strains examined. As these are well known canine pathogens, being responsible for a number of conditions from abscess formation, tonsilitis, metritis, infertility and abortion, to peracute infections in puppies, it is probable that their presence in infected ears is the result of auto-infection.

Non-haemolytic streptococci:

All strains which failed to lyse horse red cells completely were called non-haemolytic streptococci.

Whereas a preliminary classification of the haemolytic strains was afforded by the Lancefield precipitation reaction, it was not possible to differentiate the non-haemolytic strains serologically. An attempt was therefore made to distinguish various species by the usual physiological and biochemical techniques. These included the fermentation of a number of sugars, the hydrolysis of sodium hippurate and aesculin, the final pH. in glucose broth, the production of ammonia in peptone water, growth in gelatin and growth in the presence of bile salts.

The results so obtained were sufficient to distinguish the six common species, Str. faecalis, Str. bovis, Str. liquefaciens, Str. equinus, Str. mitis and Str. salivarius.

The incidence of non haemolytic streptococci in healthy dogs was shown to be, external ears 30 per cent, middle ears 14 per cent, anterior nares 46 per cent and tonsils 51 per cent.

Including 20 rectal strains which were chosen at random, a total of 91 non haemolytic streptococci were examined, details of which are included in Table 11.

TABLE 11

The species of non haemolytic streptococci in the ears, nares, tonsils and intestines of healthy dogs

Site Examined	Number of Strains Examined	Faecalis	Liquefaciens	Bovis	Salivarius	Mitis	Equinus
External ears	25	13	-	3	5	2	2
Middle ears	10	-	-	2	5	1	2
Anterior nares	16	6	1	2	5	-	2
Tonsils	20	2	2	4	8	2	2
Rectum	20	9	4	1	5	1	-
Total	91	30	7	12	28	6	8

Of the 91 non haemolytic streptococci from the different sites, Str. salivarius and Str. faecalis occurred most often, and in approximately equal numbers.

Although the incidence of Str. salivarius in healthy tonsils was lower than was anticipated, they were reasonably common in the other sites examined. It was also noticed that approximately 50 per cent of the non haemolytic strains in the healthy external ears were identified as Str. faecalis, and that neither this species nor Str. liquefaciens occurred in the middle ears.

Diphtheroid bacilli:

Gram-positive rods resembling Corynebacterium species were frequently recovered from normal material. Although a certain amount of pleomorphism was observed, the morphology of the majority of strains was that of a typical uniformly stained straight, or slightly curved, club-shaped rod.

As a general rule most strains failed to ferment carbohydrate media but the minority species, the irregularly staining forms, usually split glucose and a number of other sugars; acid being formed without visible gas. Most of the strains grew reasonably well on horse blood agar forming small (1 - 2 mm.) non haemolytic colonies, which after 48 hours' incubation at 37°C., were not unlike staphylococcal colonies. The degree of growth on Sabouraud's maltose agar, both at 37°C. and 25°C., was surprisingly good. On this latter medium both smooth and rough strains produced a characteristic biscuit coloured convex colony with a regular edge and a 'dimpled' surface. The colony of a rough strain after 21 days' incubation at 25°C. is shown on Plate 1.

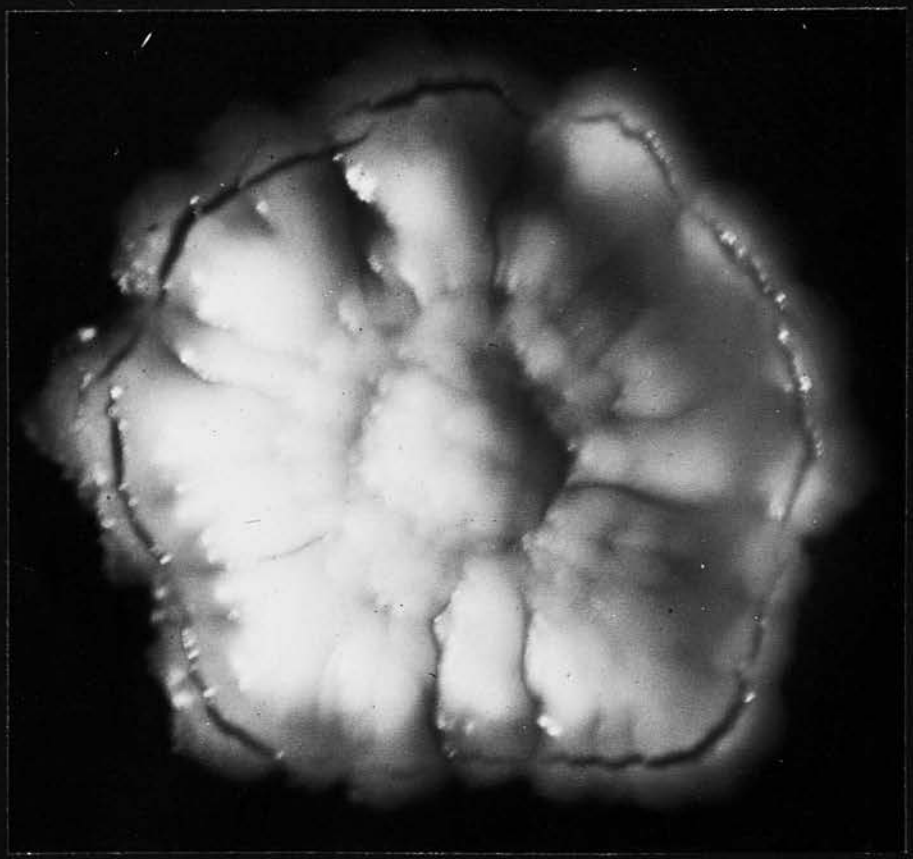
PLATE 1.

Corynebacterium species.

Surface colony, rough form, of Corynebacterium species, on the surface of a Sabouraud's maltose-agar plate after 48 hours at 37°C. and 21 days at 26°C.

Diameter of colony 3.5 mm.

Magnification x 26



The biochemical activities of this group of organisms suggested that they can be conveniently classified into the two species which closely resemble Bergey's (1948) description of Corynebacterium hoffmanni and Corynebacterium xerosis. C. hoffmanni was identified as a uniformly staining Gram-positive, club-shaped rod which did not ferment carbohydrate media, whereas C. xerosis was more pleomorphic and attacked glucose and frequently maltose and saccharose.

The frequency of both of these organisms in the normal dog is shown in the following Table:-

TABLE 12

The incidence of diphtheroid bacilli in healthy dogs

Sites Examined	Percentage Positive	Number of strains resembling:- C. hoffmanni	C. xerosis
External ears	16	8	4
Middle ears	2	1	-
Anterior nares	17	3	3
Tonsils	23	9	1
Total		21	8

Although the number of strains was small, it is perhaps significant that only one of the ten tonsil strains was identi-

identified as C. xerosis; and that most (72 per cent) were similar to C. hoffmanni.

Aerobic sporing bacilli:

In the past, there has been much confusion in the classification of this group of organisms due to the use of the name B. subtilis for a number of distinct species. As Gibson (1948) remarks, there is still widespread uncertainty concerning the relationship to B. subtilis of the organisms commonly recognised as B. mesentericus, B. vulgatus, B. pumilus and B. globigii.

The fact that there are so many named species in the genus Bacillus is due, to a large extent, to the fact that important characteristics vary from strain to strain within a species and among the variants which may be separated from a single strain.

In naming the aerobic sporing bacilli which were isolated from healthy dogs, the recommendations of Gibson (1944), Smith, Gordon and Black (1952), and Knight and Proom (1950) have been closely followed.

The criteria for differentiating the species included the size and staining reaction of the cells, the size, shape and position of the spore within the cell, the ability to ferment certain sugars especially glucose, arabinose and xylose, the production of acetylmethylcarbinol, the reduction of nitrates, the hydrolysis of gelatin and casein, and the ability of the strain to grow anaerobically.

Although the colonial form of each strain was noted, this was of little practical value due to the presence of considerable variation within the same species. B. licheniformis was the exception, however, as the presence of a 'subtilis-like' colony with fine hair-like outgrowths, which was adherent to the medium and which frequently formed watery or mucoid 'vesicles', was invariably diagnostic of this species.

Aerobic Gram-positive bacilli were recovered from 31, 6, 40 and 23 per cent respectively, of the external and middle ears, the anterior nares and the tonsils of healthy dogs. In all, 56 strains were examined in detail and classified as follows:-

TABLE 13

The identification of 56 strains of sporing aerobic bacilli, from healthy dogs

Sites Examined	Number of Strains Isolated	B. subtilis	B. cereus	B. mycoides	B. megatherium	B. circulans	B. firmus	B. pumilus (mesentericus)	B. coagulans	B. licheniformis	B. lentus
External ears	30	8	2	4	3	3	2	2	2	2	2
Middle ears	3	1	1	-	-	1	-	-	-	-	-
Anterior nares	15	5	2	4	2	2	-	-	-	-	-
Tonsil	8	4	-	2	2	-	-	-	-	-	-
Total	56	18	5	10	7	6	2	2	2	2	2

Although these results suggest that the commonest species of aerobic sporing bacilli in normal dogs are B. subtilis and B. mycoides, the figures for the external ear cultures show that no one type predominated and it would seem that members of this genus are probably present as the result of soil or other contamination.

The Pasteurella Group:

During the examination of the normal material there was evidence, in many of the cultures of tonsil swabs, of a small Gram-negative coccoid or cocco-bacillary bacterium which, when stained with Methylene blue or Leishman's stain, showed evidence of bipolar staining. The colony on horse blood agar was regular in outline, low, convex, slightly opaque or with a smooth glistening surface, between 0.5 and 1 mm. in diameter and not unlike a young Streptococcus colony. The growth, however, gave off a characteristic musty smell which is usually associated with Pasteurella septica. On nutrient agar the growth was poor, with tiny discrete entire colonies appearing after 24 hours' incubation at 37°C. In glucose broth a slight to moderate uniform turbidity was apparent in 18 hours at 37°C., but there was no evidence of a surface ring or pellicle and only occasionally was there a slight granular deposit. No visible growth occurred on MacConkey's bile salt medium after 4 days and, although stab cultures in gelatin showed a uniform but relatively

relatively poor growth along the length of the stab, there was no surface growth, or liquefaction of the medium within 6 weeks.

On solidified bovine serum slopes, a moderately heavy, confluent, raised, white or "light-ivory" growth was formed after 24 hours at 37°C.

The majority of strains reduced nitrates to nitrites although, in many cases, the reaction was very slight. Both the Methyl red and the Voges Proskauer tests were negative and ammonia was not produced in peptone water. Methylene blue was not reduced and in Litmus Milk media an initial slight acidity was the only change. None of the strains was motile and all produced detectable amounts of indole.

White mice survived the intravenous inoculation of 0.2 to 0.5 ml. of an overnight peptone water culture.

The majority of strains produced only acid in glucose, maltose, sucrose, trehalose, dextrin, galactose, laevulose and mannose. In raffinose and inositol the reactions were variable but lactose, dulcitol, mannitol, salicin, arabinose, inulin, rhamnose, xylose and sorbitol were unaffected.

In contrast to the above reactions with 18 strains, 2 strains (one from the middle ear and one from the tonsil) from different dogs, resembled Past. septica. Both were bipolar staining, Gram negative, non-motile, non capsulate, non sporing, non acid fast cocco-bacilli.

The colonial form of the Past. septica strains on blood

blood agar was generally that of the Pasteurella species above, although the diameter of the typical colony was invariably larger. The characteristic smell which one associates with Past. septica was faint but unmistakable.

Both strains produced acid, without gas, from glucose, mannitol, sucrose, trehalose, xylose, galactose, laevulose and mannose but other sugars were not attacked. Indole was produced and nitrates were reduced, but neither strain utilised citrate as a sole source of carbon. Urea was not hydrolysed, Methylene blue was unaffected and, in Litmus Milk, both strains produced a small amount of acid.

Intravenous inoculation of three white mice with 0.2 ml. of a 48-hour broth culture of each strain proved lethal within 36 hours.

TABLE 14

The incidence of Pasteurella species in healthy dogs

Number of sites examined		Pasteurella species		Pasteurella septica	
		No.	%	No.	%
External ears	70	-	-	-	-
Middle ears	50	2	(4)	1	(2)
Anterior nares	35	5	(14)	-	-
Tonsils	35	11	(31)	1	(3)
Total		18		2	

TABLE 15

Fermentation of Sugars by 18 strains of Pasteurella species

Number of Strains Pasteurella species	Glucose	Maltose	Mannitol	Inositol	Sucrose	Dextrin	Galactose	Laevulose	Mannose	Nitrates	Indole	Motility	Lethal to Mice
4	A	A	-	A	A	A	A	A	A	v.	+	-	-
1	A	A	-	A	A	-	A	A	-	+	+	-	-
8	A	A	-	-	A	A	A	A	A	v.	+	-	-
1	A	A	-	-	-	-	A	A	-	+	+	-	-
2	A	-	-	-	A	-	A	A	-	-	+	-	-
Pasteurella septica 2	A	-	A	-	A	-	A	A	A	+	+	-	+

A = Acid without gas

It will be shown later that Pasteurella species are seldom present in the infected canine ear, although the author has encountered a number of cases of otitis and cellulitis in cats where Past. septica was thought to be the pathogen concerned. The high incidence of Pasteurella species in the tonsils of normal dogs suggests either that they are non pathogenic or that, if they are capable of causing disease, they are unable to reach the external acoustic meatus by way of the eustachian tubes.

It may be correct, therefore, to assume that these organisms

organisms are commensals of the healthy canine tonsil.

The anaerobic spore-forming bacilli:

The discussion of the sporing anaerobes will be deferred until Part II when the different species in the external ears of healthy and infected dogs will be considered.

Yeasts and yeast-like organisms:

Stained films from 40 per cent of the external ears of healthy dogs showed the presence of Gram-positive yeast bodies. Of the 28 strains that were isolated in primary cultures, 25 were Pityrosporum species and 3 Candida albicans.

Pityrosporum species:

These large, strongly Gram-positive, budding cells were quite unlike the species from human sources in that they grew freely on Sabouraud's maltose or glucose agar, and on malt agar, at 37°C. and 25°C. It is emphasised that oleic acid was not necessary for the growth of these organisms on primary culture (Plate 2). The typical colony appeared as a regular, domed, buff to biscuit-coloured structure of about 2 mm. in diameter and of a dryish and somewhat friable consistency. There was no evidence of a pseudo-mycelium.

On 5 per cent horse blood agar the degree of growth was poor and the colonies, which were non haemolytic, rarely attained a diameter of 0.5 mm.

PLATE 2.

Pityrosporum species.

Primary culture, from an ear swab, of Pityrosporum species on Sabouraud's maltose-agar after 48 hrs. at 37°C. Showing the heavy growth of yeast colonies on mycological media without the addition of oleic acid.

Magnification - actual size.

In an attempt to differentiate between the 25 Pityrosporum strains recourse was made to bacteriological techniques. Unfortunately, it was found that they all gave negative reactions to sugar fermentation, indole, M.R., V.P., nitrate reduction, citrate utilisation, Methylene blue and Litmus Milk, gelatin, casein and urea hydrolysis and the production of hydrogen sulphide. This may have been due, in part, to the poor growth obtained in many of the test media but the addition of oleic acid, olive oil, whale oil and various antibiotics produced no appreciable improvement.

Because of the failure to classify the strains by biochemical methods, each strain was examined under different conditions in a wide variety of media in an attempt to induce some peculiarity or morphological change in the process of vegetative reproduction which might, as with many other yeasts, serve as a basis for classification.

Pityrosporum species did not appear to be present in the other tissues examined.

Candida albicans:

In addition to the three strains of C. albicans from the external ears, two strains were recovered from the tonsils of healthy dogs.

Candida species were recognised as yeast-like bodies which reproduced by budding but did not form asci or aerial hyphae. The colonies on maltose or malt agar were raised, white to ivory

ivory in colour, moist and of a creamy consistency. On horse blood agar the colonies were regular, markedly convex, 2 - 3 mm. in diameter and frequently showed a characteristic mycelial fringe about the entire colony. Pseudomycelia were frequently observed.

All the strains produced 'surface bubbles' in acid glucose broth after 48 hours at 37°C., and fermented glucose, maltose, laevulose and dextrin to form both acid and gas. Acid, but not gas, was produced in sucrose and galactose, whereas mannitol, inulin and lactose were not fermented.

Corn-meal agar and carrot-plug cultures failed to show the formation of chlamydospores or asci, but overnight nutrient broth cultures proved lethal to rabbits and white mice when inoculated intravenously.

The incidence of yeasts and yeast-like organisms is summarised in the following Table.

TABLE 16

The incidence of Pityrosporum and Candida species in healthy dogs

Sites and number examined		Number of positive swabs and the species of yeasts present	
		Pityrosporum	Candida
External ears	70	25 (36%)	3 (4%)
Middle ears	50	-	-
Anterior nares	35	-	-
Tonsils	35	-	2 (6%)

Fungi:

The commonest fungi in the external ears of healthy dogs were one or other of the many Aspergillus species. Aspergillus fumigatus has been described by Ainsworth (1954) as an ubiquitous mould of soil and compost which is usually and unequivocably a saprophyte, although at other times it is equally clearly a virulent and lethal pathogen. The same author found A. fumigatus to be the commonest fungus, in his survey of fungoid infections of animals in Britain (Ainsworth, 1954).

There can be little doubt, therefore, that the fungi present in the external auditory canals of these normal dogs were other than saprophytes. Whereas this well-known fungus was easily recognised by its colonial characteristics and, in particular, by the arrangement of the sterigmata in a single row around the conidiophore vesicle, considerable difficulty was experienced in attempting to identify accurately other species of 'higher fungi'. This was particularly true of the Penicillium family. Throughout this paper a strain will be given the family name only, if there was doubt as to its proper species.

The Phycomycetes, which produced characteristic colonies on the usual mycological media, were readily identified as Mucor, Rhizopus or Absidia species by means of agar slide-culture techniques. These methods were invaluable for demonstrating, not only the morphology of the sporangium and columella, but also the presence and the position of rhizoids.

Fungi were present in 11 and 29 per cent respectively of the external ear and nasal swabs. Cultures from the middle ears and tonsils proved negative. The complete absence of moulds in the tonsils of healthy dogs was unexpected.

The detailed results can be summarised as follows:-

TABLE 17

Fungi in the ears and nares of healthy dogs

Sites and Number Examined		SPECIES							
		Aspergillus	Penicillium	Mucor	Absidia	Rhizopus	Geotrichum	Botrytis	Cladosporium
External ears	70	4	3	-	2	3	1	-	1
Anterior nares	35	3	4	2	-	-	-	1	2

The species of fungi and the number of strains recovered are contained in the following list:-

External ear	Nose	Identity of the Fungus
2	2	Aspergillus fumigatus
1	-	Aspergillus terreus
1	1	Aspergillus species
2	-	Penicillium expansum
1	4	Penicillium species
3	-	Rhizopus nigricans
1	-	Absidia ramosa
1	-	Absidia corymbifera
1	-	Geotrichum (?) candidum
1	2	Cladosporium herbarum
-	1	Mucor racemosus
-	1	Mucor species
-	1	Botrytis cinerea

The relatively large number of different species compared with the small total number of strains recovered, also suggested that these moulds were saprophytic. There was no evidence of dermatophytes, although many of the swab-inoculated media were incubated for prolonged periods at 25° and 37°C.

PART I - SUMMARY:

Material was examined from the external and middle ears, the anterior nares, the tonsils and the recta of 35 clinically healthy dogs.

Although Pr. mirabilis was isolated from 37 per cent of the rectal swabs, neither it nor Ps. aeruginosa was recovered from the ears, nares or tonsils of healthy dogs; both species are said to be frequently associated with otitis. Only 3 strains of E. coli, one of which was probably of faecal origin, were isolated from the external ears, whereas they were frequently present in the other sites examined.

Staphylococci were present in 54 per cent of the external ears, 46 per cent of the strains being coagulase positive, and in 18 per cent, 71 per cent and 49 per cent of the middle ears, nares and tonsils respectively, of healthy dogs. Haemolytic streptococci, on the other hand, were rarely seen in the external ears, being commonest in the tonsils (34 per cent) and the anterior nares (11 per cent). Most of the strains were classified in Lancefield's Group G., although Group M strains were not uncommon in the tonsil and nares swabs.

Other common bacterial species in the healthy meatus were anaerobic spore-forming bacilli, most of which were identified as Cl. welchii, type A. These organisms, however, were rarely seen in direct smears, although they were isolated from no fewer than 44 per cent of the external ears.

Very few strains of typical Past. septica were found in healthy dogs, but an unidentified Pasteurella species was isolated from 31 per cent and 14 per cent respectively of the tonsil and nares swabs. Other commonly occurring bacteria included non haemolytic streptococci, diphtheroid bacilli and the B. subtilis group of organisms.

Apart from Pityrosporum species, which were isolated from 34 per cent of the external ears, but from no other site, yeasts and fungi were rarely seen.

PART II

The bacterial and fungal flora of the ears of dogs affected with otitis; the swabs being taken before the commencement of treatment

Part II is devoted to a study of the flora of infected ears, the results being compared with those of the ears, nose and tonsils of clinically healthy dogs.

Material was obtained, prior to treatment, from 363 dogs of which 160 were cases of bilateral otitis and 203 were from dogs affected in one ear only. The case history which was kept of each dog included details such as age, sex, breed, conformation of the ears, the dog's general state of health, previous illnesses, the presence of ectoparasitic infestation, skin lesions and any other abnormality which may have predisposed to the condition.

Although many of the dogs were not pure bred, most of them showed the characteristics of a particular breed and were identified accordingly. By this method, the dogs affected with otitis were classified into 32 different breeds as is shown in Table 18.

TABLE 18

Dogs suffering from otitis

Breed	M	F	Total	Breed	M
+ Alsatian	17	5	22	"Terrier Types"	
+ Collie	22	7	29	+ Scotch terrier	9
+ Bulldog	3	1	4	+ Cairn terrier	5
+ Corgi	2	1	3	+ Fox terrier	9
+ Chow	2	1	3	+ West Highland	5
+ Samoyed	1	-	1	+ Bull terrier	4
+ Greyhound	1	-	1	Border terrier	5
Spaniel	82	40	122	Airedale	4
Labrador	16	16	32	Sealyham	-
Boxer	4	7	11	Yorkshire terrier	-
Poodle	7	4	11	Bedlington terrier	1
Dachshund	3	3	6	Dandie Dinmont	1
Pyrenean mountain	3	-	3	Kerry blue terrier	-
English sheepdog	-	2	2		
Irish Setter	1	1	2	Mongrels	
Dalmatian	2	-	2	"Cross" terrier	16
Afghan	1	1	2	Unidentified	1
Pekingese	-	1	1		
Pug	1	-	1	TOTAL	60
Total	168	90	258	GRAND TOTAL	228

The average age was 5 years and 3 months. The symbol "+" indicates the dogs with 'Prick' or 'Semi-prick' ears, of which there were 1 The remaining 248 dogs had 'drop' ears. The letters M and F re male and female dogs respectively.

TABLE 18

Dogs suffering from otitis

Breed	M	F	Total	Breed	M	F	Total
+ Alsatian	17	5	22	"Terrier Types"			
+ Collie	22	7	29	+ Scotch terrier	9	8	17
+ Bulldog	3	1	4	+ Cairn terrier	5	7	12
+ Corgi	2	1	3	+ Fox terrier	9	1	10
+ Chow	2	1	3	+ West Highland	5	4	9
+ Samoyed	1	-	1	+ Bull terrier	4	-	4
+ Greyhound	1	-	1	Border terrier	5	2	7
Spaniel	82	40	122	Airedale	4	-	4
Labrador	16	16	32	Sealyham	-	4	4
Boxer	4	7	11	Yorkshire terrier	-	1	1
Poodle	7	4	11	Bedlington terrier	1	-	1
Dachshund	3	3	6	Dandie Dinmont	1	-	1
Pyrenean mountain	3	-	3	Kerry blue terrier	-	1	1
English sheepdog	-	2	2				
Irish Setter	1	1	2	Mongrels			
Dalmatian	2	-	2	"Cross" terrier	16	14	30
Afghan	1	1	2	Unidentified	1	3	4
Pekingese	-	1	1	TOTAL	60	45	105
Pug	1	-	1				
Total	168	90	258	GRAND TOTAL	228	135	363

The average age was 5 years and 3 months. The symbol "+" indicates the dogs with 'Prick' or 'Semi-prick' ears, of which there were 115. The remaining 248 dogs had 'drop' ears. The letters M and F represent male and female dogs respectively.

The incidence of the more frequently occurring micro-organisms in the ears of dogs suffering from otitis externa

In addition to the routine inoculation of the various cultural media (page 13), the exudate from the infected meatus was smeared and stained by Gram's method. These preliminary examinations provided a reasonably accurate indication of the frequency of the different micro-organisms in the infected ears; the more important species being Pseudomonas, Proteus, coliforms, staphylococci, streptococci, 'diphtheroids', aerobic sporing bacilli and Pityrosporum.

The anaerobic sporing bacilli, on the other hand, would appear to occupy rather a peculiar position as they were frequently present in small numbers in primary culture, but were seldom seen in stained smears of the exudate. This, and the fact that an almost identical picture was obtained during the study of the flora of healthy ears, suggested that the presence of sporing anaerobes in the external auditory canal was entirely fortuitous, their presence being due to contamination from an external source. It was decided, therefore, to include a discussion of the anaerobes after that of the aerobic species.

The findings of the bacteriological examinations of otitic material are briefly outlined in Table 19.

TABLE 19

The incidence of aerobic bacteria in otitic material

Number of ears examined 523

	Pseudomonas	Proteus	Coliforms	Beta haemolytic streptococci	Alpha haemolytic streptococci	Non haemolytic streptococci	Staphylococci	Diphtheroids	Subtilis group
No. of ears infected	67	82	68	95	33	100	321	89	62
Percentage No. infected	13	16	13	18	6	19	61	17	12

The results in Table 19 (which are reported in detail in Appendix 3) clearly show that in cases of otitis, the incidence of six of the seven commonly occurring groups of organisms is approximately equal. This was particularly true of the Gram-negative organisms Ps. aeruginosa, Pr. mirabilis and the coliforms which were recovered respectively from 13, 16 and 13 per cent of infected ears .

In marked contrast is the incidence of staphylococci, most of which were non-pigmented, coagulase-producing strains. The figure of 61 per cent was considered to be of sufficient interest to justify a thorough investigation of as many strains as possible, in the hope that the information gained would throw some light on the part played by staphylococci in the aetiology of otitis.

The relative frequency of the different bacterial species in otitic material is shown in Table 20.

TABLE 20

The relative frequency of occurrence of bacterial species in otitic material

Number of ears examined - 523

Species	Ears in which the species was predominant		Pseudomonas	Proteus	Coliforms	Beta haemolytic streptococci	Alpha haemolytic streptococci	Non haemolytic streptococci	Staphylococci	Diphtheroids	Subtilis group
	No.	%.									
Pseudomonas	23	(34)	-	20	17	15	2	7	18	14	7
Proteus	20	(24)	20	-	13	15	3	18	35	18	9
Coliforms	7	(10)	17	13	-	20	5	22	53	18	12
Beta haemolytic streptococci	8	(8)	15	15	20	-	5	10	67	27	14
Alpha haemolytic streptococci	10	(30)	2	3	5	5	-	2	15	0	3
Non haemolytic streptococci	5	(5)	7	18	22	10	2	-	82	18	10
Staphylococci	101	(32)	18	35	53	67	15	82	-	65	37
Diphtheroids	8	(9)	14	18	18	27	0	18	65	-	22
Subtilis group	10	(16)	7	9	12	14	3	10	37	22	-

NOTE: '%' The number of ears in which a particular organism predominated was expressed as a percentage of the number of ears from which the organism was isolated and NOT of the total number of ears examined.

These results suggest that the commonest "pathogenic" organisms in external otitis were Pseudomonas, Proteus and staphylococci, of which only the staphylococci were found in healthy ears.

TABLE 21

The incidence of the more frequently occurring bacteria in healthy and infected canine ears

Bacterial species	Otitic material (523 ears)		Healthy material (70 ears)	
	No.	%	No.	%
Pseudomonas	67	(13)	-	-
Proteus	82	(16)	-	-
Coliforms	68	(13)	4	(6)
Haemolytic streptococci	95	(18)	2	(3)
Non haemolytic streptococci	133	(25)	21	(30)
Staphylococci	321	(61)	39	(54)
Diphtheroids	89	(17)	11	(16)
Subtilis group	62	(12)	22	(31)

The above results, unlike those in Table 20, show the incidence, in external ears, of the more frequently occurring bacteria, whether or not they were the predominant species present. Of those that were frequently isolated from both healthy and infected ears, staphylococci and 'diphtheroids' were commoner in otitis, whereas non haemolytic streptococci and

and aerobic sporing bacilli were more frequent in 'normal' ears. Most of the "coliforms" and haemolytic streptococci, and all of the Pseudomonas and Proteus strains were isolated from otitic material which suggests that they are the organisms mostly concerned with the clinical forms of otitis. The results also suggest that the Gram-negative species are contaminants and that staphylococci and 'diphtheroids' are commensals of the external meatus.

The nature of the discharge in the external ears of dogs suffering from otitis

This survey is based on material from 371 untreated ears, from the first 268 clinically affected dogs.

TABLE 22

The nature of the discharge in affected ears

Type of discharge	Ears affected	
	No.	%
Scanty	44	(11)
Moderate	195	(53)
Abundant	132	(36)
Moist	259	(70)
Dry	112	(30)
Pale/yellow	24	(7)
Yellow	61	(16)
Yellow/brown	70	(19)
Red/brown	90	(24)
Brown/black	126	(34)

Although these figures show that the discharge in most cases of external otitis was moist, moderately abundant and of a brownish colour, it became evident that the bacterial findings were related to the nature of the discharge in the affected ear. As the presence of any such correlation would be of clinical value, the bacterial flora was compared with the type of discharge in the infected meatus. (Table 23).

TABLE 23

The correlation of the bacterial findings with the nature of the discharge

Number of ears examined 371

Type of Discharge		Pseudomonas	Proteus	Coliforms	Staphylococci	Beta and alpha streptococci	Non haemolytic streptococci	Subtilis group	Diphtheroids	No organism recovered	Fungi and Yeasts
Scanty		1	0	0	21	5	4	1	5	7	24
Moderate		5	12	18	139	45	40	25	22	0	115
Abundant		36	33	40	87	46	34	23	36	1	52
Pale	Moist	8	5	2	8	3	2	4	3	0	0
	Dry	0	0	0	6	2	1	0	0	4	1
Yellow	Moist	20	30	16	26	19	21	4	10	0	1
	Dry	1	6	1	3	4	4	1	3	1	1
Yellow Brown	Moist	4	7	15	47	14	10	11	10	0	13
	Dry	0	0	0	5	3	6	0	2	2	4
Red Brown	Moist	6	6	15	51	17	16	9	15	0	34
	Dry	0	0	4	15	5	4	4	3	0	21
Brown Black	Moist	3	1	5	59	20	10	12	14	0	69
	Dry	0	0	0	27	9	4	4	3	1	47
Total number of ears affected		42	55	58	247	96	78	49	63	8	191

The figures in Table 23 show that the Gram-negative organisms Pseudomonas, Proteus and coliforms, were usually associated with an abundant discharge which tended to be moist and light in colour. Staphylococci and yeasts, on the other hand, were commonest in the dark-coloured exudate which was also drier and less copious. There was, however, little or no correlation between the nature of the discharge and the presence of streptococci, 'diphtheroids' or the subtilis group of organisms.

If it be accepted that Pseudomonas, Proteus, staphylococci and Pityrosporum are the most important species in external otitis, then the type of discharge may suggest, not only the identity of the pathogen concerned, but also the most suitable method of treatment for each individual case.

The more frequently occurring micro-organisms in both healthy and infected dogs

This section will deal with the incidence and the main characteristics of the more frequently occurring micro-organisms in both healthy and infected dogs.

Pseudomonas:

Members of this genus, which were readily identified by their ability to grow well on ordinary media with the production of at least one water-soluble pigment, were invariably aerobic, non-sporing, actively motile, Gram-negative rods. The term Ps. aeruginosa was used to include all species within the genus which produced a chloroform soluble pigment, pyocyanin, or which, in the absence of this pigment, grew at 42°C. Haemolysis of horse red cells, liquefaction of solidified bovine serum and gelatin, delayed hydrolysis of urea and the absence of levan production on sucrose agar were accepted as additional characteristics.

Although Pseudomonas species did not appear to be present in the ears of healthy dogs, they were isolated from 67 (13 per cent) of the 523 infected ears. In most cases the primary growth was very heavy, not less than 34 per cent of the aeruginosa strains being in pure culture. This, and the fact that the organisms were usually very numerous in the direct smears of otitic material, suggested that they may be of pathogenic

pathogenic importance.

The main characteristics of the 67 otitic strains are shown in Table 24.

TABLE 24

Number of 'normal' ears infected with Pseudomonas - 0

Number of otitic ears infected with Pseudomonas - 67

	Number of strains	
	+	-
Motility	67	0
Indole production	0	67
Nitrate reduction	1	66
Gelatin liquefied	66	1
Serum digested	66	1
Catalase produced	67	0
V.P.	0	67
M.R.	0	67
Citrate utilised	67	0
Growth at 42°C.	66	1
Growth anaerobically	1	66
Haemolysis of horse cells	49	18
Methylene blue reduced	67	0
Delayed urea hydrolysis	66	1
Levan produced	0	67
Pyocyanin produced	35	32
Fluorescin produced	57	10
≠ Acid only from -		
Glucose	67	0
Mannitol	65	2
Sucrose	8	59
Arabinose	66	1
Trehalose	28	39
Xylose	66	1
Glycerol	67	0
Galactose	67	0
Laevulose	67	0
Mannose	67	0

NOTE: (≠) = In nitrogen-free sugar media

All the Pseudomonas strains from infected ears were identified as Ps. aeruginosa. Most of them reduced nitrates to nitrites and liquefied gelatin and solidified bovine serum slopes, but none of the strains produced levan on sucrose agar. Although the fermentation of ordinary fluid carbohydrate media was not a feature of the Pseudomonas species of canine origin, they all produced acid, but not gas, from glucose, glycerol, galactose, laevulose and mannose and in a number of cases from mannitol, sucrose and trehalose, when tested in the nitrogen-free sugar medium of Liu (1952).

In most other respects the Ps. aeruginosa dog strains were typical of those from other sources.

Proteus:

The members of this group which conformed to the definition of Enterobacteriaceae and were motile rods which rapidly hydrolysed urea were recovered from 82 (16 per cent) of the external ears. Of these, 79 were identified as Pr. mirabilis, 2 as Pr. vulgaris and 1 as an atypical Pr. morganii. Pr. rettgeri was not present in any of the ears examined.

The incidence of Proteus species in the ears, nose, tonsils and rectum of healthy dogs and in the ears of dogs affected with otitis is shown in the following Table.

TABLE 25

The incidence of species of Proteus in healthy and infected dogs

	Affected ears	Healthy Dogs. Ears	Nose	Tonsils	Rectum
No. examined	523	70	35	35	30
No. positive	82 (16)	-	-	2 (6)	14 (47)
Pr. mirabilis	79 (15)	-	-	-	11 (37)
Pr. vulgaris	2 (0.4)	-	-	2 (6)	3 (10)
Atypical Pr. morganii	1 (0.2)	-	-	-	-

Numerals in brackets show the percentage of ears infected

The relatively low incidence of Proteus in infected ears (16 per cent) is in marked contrast to the severity of the clinical reaction with which it is associated. It seemed as if the ratio of Pr. mirabilis to Pr. vulgaris in infected material might be of importance, as the figure (which was approximately 30 to 1) was quite different from that of the rectal swabs of healthy dogs, when the mirabilis/vulgaris ratio was almost 7 to 2. Although he did not state the incidence of the different species of Proteus in the intestines of healthy dogs, Phillips (1955) found that of 125 dog strains, most of which were from pathological specimens, 118 were Pr. mirabilis and 5 were Pr. vulgaris: a ratio of approximately 24 to 1. As the above findings are in close

close agreement, it seems probable that Pr. mirabilis is the commonest species of Proteus in pathological processes in dogs.

Only those reactions which were necessary for the identification of the species are included in the following Table.

TABLE 26

The characteristics of Proteus species from infected ears

Number of strains examined - 82

Fermentation of -	No. of strains reacting positively: Atypical Pr. morganii	Pr. vulgaris	Pr. mirabilis	Total
Glucose	1	2	79	82
Maltose	-	2	-	2
Sucrose	-	2	62	64
Trehalose	-	2	79	81
Salicin	-	2	16	18
Galactose	1	2	78	81
Glycerol	1	1	79	81
Xylose	-	2	78	80
Laevulose	1	2	77	80
Mannose	1	-	-	1
Other sugars	-	-	-	-
Motility	1	2	77	80
Swarming	-	2	77	79
Indole	1	1	14+	15+
Hydrogen sulphide	1	2	79	82
Urea	1	2	79	82
Gelatin	-	2	78	80
Solid serum	-	2	76	78
Number of strains examined	1	2	79	82

+ The majority of these 14 strains of Pr. mirabilis produced traces of indole-acetic acid irregularly.

Pr. mirabilis:

The above results show that most of the Pr. mirabilis strains were fairly typical in their behaviour. Although none of them fermented maltose, a fairly high percentage (22 per cent) failed to ferment sucrose within 21 days, while an unexpectedly large number of strains fermented salicin (20 per cent). Only one strain failed to ferment galactose and xylose, two strains did not attack laevulose, but they all produced hydrogen sulphide and rapidly decomposed urea.

Two strains were atypical in that they were non-motile and did not swarm on 1.5 per cent nutrient agar.

Pr. vulgaris:

Only two strains were identified as Pr. vulgaris, both being very similar in most respects. Although their action on glycerol was poor, one strain produced acid and gas in 6 days, whereas the other produced only acid in 48 hours without evidence of visible gas after 21 days. One of the strains, which was otherwise a typical Pr. vulgaris, failed to produce indole.

Atypical Pr. morganii:

This motile, non-swarming Gram-negative rod was included in the Proteus group by virtue of the fact that it produced acid with gas in glucose and hydrolysed urea within 12 hours. It also produced hydrogen sulphide in lead acetate agar and in peptone water cultures. Although it resembled Pr. mirabilis

Pr. mirabilis by failing to ferment maltose, it did not attack sucrose, trehalose or xylose; nor was it proteolytic. These features, plus the fact that it was the only strain to ferment mannose, placed it as an atypical Pr. morganii, rather than an atypical Pr. mirabilis.

Advantage was taken of the work of Story (1954) and Krikler (1953) to distinguish antigenically identical strains of Pr. mirabilis, by means of the Dienes' phenomenon. (Dienes, 1946). This is discussed in detail in Part VI.b.

Of the 79 mirabilis strains from infected ears, 4 were untypable, due to an alteration of phase, and 8 remained untyped as they did not fall into one of the 14 identified groups. The fact that 32 (41.5 per cent) of the typable strains were antigenically identical suggested the possibility of a common source of Proteus infections in dogs. In only two instances were antigenically dissimilar mirabilis strains recovered from different ears of the same dog.

The coliform group:

Coliform organisms, which included members of the genera Escherichia, Klebsiella, Cloaca and Hafnia, were present in 68 (13 per cent) of the 523 infected ears. This approximates to the incidence of the other important Gram-negative bacilli, Proteus and Pseudomonas, which were present in 16 and 12 per cent respectively of infected ears.

TABLE 28

The incidence of 'coliforms' in normal and affected ears

	Otitic Dogs	Healthy Dogs			
	Infected ears	Outer ears	Middle ears	Nares	Tonsils
No. examined	523	70	50	35	35
No. positive	68	4	6	5	22
Percentage positive	(13)	(6)	(12)	(14)	(63)

These findings suggest that the tonsils and, to some extent, the middle ears and nares may serve as a possible source of infection in cases of external otitis. As it has already been shown (Part I) that most E. coli strains are probably of faecal origin, it became necessary to ascertain the incidence of faecal strains in otitic material.

Of the 68 strains examined, 58 (85 per cent) were identified as E. coli, 2 as Klebsiella species, 1 as E. freundii, 5 as Cloaca cloacae and 2 as Hafnia species. It was further shown that 53 (95 per cent) of the E. coli strains were probably of faecal origin as they were indole and M.R. positive but V.P. and citrate negative. The distribution of 'faecal coliforms' in healthy and affected dogs is shown in Table 29.

TABLE 29

The distribution of typical *E. coli*, of faecal origin, in healthy and affected dogs

Number of strains examined - 108

	Infected external ears	HEALTHY DOGS. External ears	Middle ears	Nares	Tonsils	Rectum
No. of coliforms examined	58	3	5	4	15	23
No. of faecal *E. coli*	53	1	5	4	11	22
% of faecal *E. coli*	(95)	(33)	(100)	(100)	(73)	(96)

The results in Table 29 show that coliform organisms were not normally present in healthy external ears. The fact that the majority of strains from other sites in normal animals, and from the ears of dogs affected with otitis, were of faecal origin is very similar to the condition in man. Not only does Stewart (1951) emphasise the importance of coliform organisms in cases of human otitis, but he suggests that they are usually present as the result of auto-infection from the gut.

A summary of the more important characters of the *E. coli* strains from otitic material is included in the following Table.

TABLE 30

E. coli strains from otitic material

Fermentation of -	No. of strains
Lactose	58
Dulcitol	52
Inositol	-
Sucrose	29
Salicin	50
Arabinose	57
Indole	58
Motility	40
M.R.	58
V.P.	-
Citrate	55
Nitrate	58
Eijkman test	58

Staphylococci:

As staphylococci were found to be the commonest bacterial species in the material from infected ears, it was decided to examine the characteristics of as many strains as possible in an attempt to ascertain their pathogenic significance.

No fewer than 321 (61 per cent) of the 523 ears were infected with staphylococci of which the first 200 strains were examined in detail.

By subdividing the staphylococci according to their coagulase activity, which is generally held to be an indication of pathogenicity, it was possible to compare the strains from infected ears with those from the ears, nose and tonsils of healthy dogs.

TABLE 31

The staphylococci from normal and affected dogs

		Healthy Dogs				
		Infected ears	External ears	Middle ears	Nose	Tonsils
No. of strains examined		200	76	12	25	20
Coagulase +ve	No.	156	35	2	17	9
	%	(78)	(46)	(17)	(68)	(45)
Coagulase -ve	No.	44	41	10	8	11
	%	(22)	(54)	(83)	(32)	(55)

Coagulase producing staphylococci were present in just over half of the healthy external ears and tonsils, whereas non-pathogenic strains predominated in the middle ears. The nasal carriage of pathogenic staphylococci in healthy animals was surprisingly high (68 per cent) which may be, as suggested by Rountree (1956) due to the fact that the majority of so-called 'normal' dogs were surgical in-patients in this School.

A number of other features, apart from coagulase activity, were thought to be of value in the differentiation of staphylococci. These included haemolysin production, the ability to liquefy gelatin and solidified serum, and the action on lactose, maltose and mannitol sugars. Other reactions, including the inability of most coagulase positive dog staphylococci to form pigment, will be discussed in Part VI.d.

The results showed that a definite correlation existed

existed between the pathogenic staphylococci from normal dogs and those from the ears of dogs affected with otitis, as is shown in the following Table.

TABLE 32

A comparison of the coagulase producing staphylococci from normal and affected dogs

Source and number of strains examined												
Healthy dogs				Otitis								
Nose	Tonsil	Middle ears	External ears	External ears	α haemolysin	β haemolysin	δ haemolysin	Gelatin liquefaction	Serum liquefaction	Lactose	Maltose	Mannitol
1	-	-	1	16	+	+	+	+	+	+	+	+
-	-	-	2	17	+	+	+	+	+	+	+	0
4	1	-	5	3	+	0	+	+	+	+	+	+
1	2	-	2	2	+	0	+	+	+	+	+	0
6	2	-	6	58	0	+	+	+	+	+	+	+
4	3	2	16	34	0	+	+	+	+	+	+	0
-	-	-	2	1	0	+	+	+	+	+	0	0
-	-	-	1	1	0	+	0	+	+	+	0	+
1	-	-	-	-	0	+	+	+	+	0	+	0
-	1	-	-	-	0	+	+	+	0	+	+	-

NOTE: The remaining 24 coagulase positive staphylococci from affected animals are not included in this Table as they formed no fewer tham 16 subdivisions, none of which is common to the strains from normal dogs.

These results suggested that potentially pathogenic strains from different sites in normal dogs were similar to the predominant types of pathogenic staphylococci in otitic material. It is probable, therefore, that in many cases of 'staphylococcal otitis' in dogs, the infection in the external meatus is the result of multiplication of the normal flora, which might occur when conditions in the ear are most favourable to bacterial multiplications, e.g. the presence of abrasions, irritation by foreign bodies, eczema or other lesions.

It was not possible to form a similar comparison with the coagulase negative staphylococci as only a few strains gave identical reactions. Nevertheless, a number of interesting observations may be made by summarising the findings as in Table 33.

It will be apparent, from these results, that coagulase negative staphylococci which produced only beta haemolysin were not recovered from the anterior nares, tonsils or middle ears of clinically healthy dogs. On the other hand, although 17 per cent of the strains from healthy external ears did so, this type of Staphylococcus was much more common in the outer ears of affected animals (61 per cent). It is difficult to account for this difference between non pathogenic staphylococci from normal and affected ears as it has already been suggested that the degree of growth in the infected meatus was the result of activation of the existing flora.

TABLE 33

A summary of the characteristics of coagulase negative staphylococci from healthy and affected dogs

Source and number of strains examined					REACTION						
Healthy dogs				Otitis							
Nose	Tonsil	Middle ears	External ears	External ears	β. haemolysin only	Haemolysin not produced	Liquefaction of gelatin	Liquefaction of serum	Lactose	Maltose	Mannitol
-	-	-	7	27	+						
8	11	10	34	17		+					
5	5	5	22	21			+				
-	3	-	13	20				+			
4	3	2	26	33					+		
7	7	4	32	27						+	
4	2	3	10	24							+
8	11	10	41	44 = Total number of strains examined from each site							

The main differences between pathogenic and non pathogenic staphylococci may be summarised as follows:- The coagulase positive canine strains produced both beta and delta haemolysins but rarely alpha toxin. Although most strains were actively proteolytic and produced acid from lactose and maltose, many failed to ferment mannitol. The coagulase negative strains did not form either alpha or delta haemolysins, although a few produced beta toxin. Their fermentative activities were also less marked than those of the coagulase positive strains.

It will be shown later (Part VI.d.) that coagulase positive canine staphylococci differed from those from human and other animal sources.

The haemolytic streptococci:

Beta haemolytic streptococci were isolated from 95 (18 per cent) of the 523 affected ears, the percentage incidence in healthy and affected dogs being as follows:-

	HEALTHY DOGS			
Infected external ears	External ears	Middle ears	Anterior nares	Tonsils
18%	3%	6%	11%	34%

These figures show that, although the nasal carriage of haemolytic streptococci was not uncommon, the majority of strains were isolated from the tonsils of healthy dogs. Moreover, as beta haemolytic streptococci were present in 6 per cent of the

the normal middle ears, it is possible that the main source of infection is the tonsils, from which the infection probably travels by way of the eustachian canal to the middle and external ears.

All strains were initially classified by Lancefield's precipitation techniques.

TABLE 34

The haemolytic streptococci from healthy and affected animals

Source Strains	Number of Strains	LANCEFIELD'S GROUPS							
		G	L	C	D	B	M	A	H
External otitis	95	56 (59)	17 (18)	15 (16)	4 (4)	1 (1)	2 (2)	-	-
Normal -									
External ears	2	1	-	1	-	-	-	-	-
Middle ears	3	1	-	-	-	-	2	-	-
Nose	6	3	2	-	-	-	1	-	-
Tonsils	18	9	-	3	-	1	5	-	-
Rectum	5	3	-	-	2	-	-	-	-
All normal sites	34	17 (50)	2 (6)	4 (12)	2 (6)	1 (3)	8 (24)	-	-

Numerals in brackets are percentages of the total number of strains examined

These results show the order of frequency of haemolytic streptococci in external otitis to be Groups G. L. and C., whereas in the ears, nose, tonsils and rectum of normal dogs it was Groups, G. M. and C.

Although the pathogenic significance of Group M strains is in doubt, the fact that only 2 strains were present in the 523 affected ears suggests that they are of little importance in canine otitis.

The potentially pathogenic Groups G. and C. were equally prevalent in both healthy and affected tissues whereas Group L streptococci were rarely seen in normal dogs.

Details of the biochemical reactions of the haemolytic streptococci are discussed in Part VI.e. but a summary is included in Table 35.

TABLE 35

The biochemical reactions of the haemolytic streptococci from infected external ears

	LANCEFIELD'S GROUPS											
	G		L		C		D		B		M	
	+	-	+	-	+	-	+	-	+	-	+	-
Na. Hippurate	2	54	0	17	1	14	0	4	1	0	0	2
Aesculin	54	2	10	7	14	1	4	0	0	1	0	2
Arabinose	6	50	0	14	0	15	0	4	0	1	0	2
Maltose	54	2	17	0	13	2	4	0	1	0	2	0
Sucrose	56	0	17	0	15	0	4	0	1	0	0	2
Lactose	52	4	10	7	14	1	4	0	1	0	2	0
Trehalose	23	33	17	0	7	8	4	0	1	0	1	1
Raffinose	4	52	0	17	0	15	1	3	0	1	0	2
Inulin	0	56	0	17	0	15	0	4	0	1	0	2
Glycerol	11	45	2	15	2	13	4	0	1	0	1	1
Mannitol	2	54	0	17	0	15	4	0	0	1	0	2
Sorbitol	0	56	0	17	3	12	4	0	0	1	0	2
Salicin	50	6	6	11	3	12	4	0	1	0	0	2
Methylene blue	7	49	0	17	1	14	4	0	0	1	0	2
Litmus Milk -												
Acid	53	3	17	0	7	8	4	0	1	0	2	0
Clot	51	5	9	8	7	8	4	0	1	0	0	2
Reduction	30	26	6	11	5	10	4	0	1	0	0	2
Growth on -												
MacConkey	0	56	0	17	0	15	4	0	0	1	0	2
Gelatin -												
Growth on	47	9	17	0	v.	v.	4	0	1	0	0	2
Liquefaction	0	56	0	17	0	15	4	0	0	1	0	2
Number of strains	56		17		15		4		1		2	

Although it was not possible to distinguish the various groups biochemically, members of Groups G. L. C. and D. were found to give the following general reactions:-

Group	Trehalose	Glycerol	Salicin	Lactose	Aesculin	Mannitol	MacConkey
G	v.	v.	+	+	+	-	-
L	+	-	v.	v.	v.	-	-
C	v.	-	-	+	+	-	-
D	+	+	+	+	+	+	+

Non haemolytic streptococci:

The other members of this group were classified as alpha or non haemolytic streptococci according to the type of growth on 5 per cent horse blood agar plates.

Of the 523 infected ears, alpha haemolytic strains were recovered from 33 (6 per cent) and non haemolytic strains from 100 (19 per cent). As the majority of these organisms could not be typed serologically, they were identified by the usual physiological and biochemical techniques.

The incidence of alpha and non haemolytic streptococci in normal and affected animals is shown in Table 36.

TABLE 36

The incidence of alpha and non haemolytic streptococci in normal and affected animals

	AFFECTED DOGS	HEALTHY DOGS			
	External ears	External ears	Middle ears	Nose	Tonsils
No. examined	133	21	7	16	18
% present	(25)	(30)	(14)	(46)	(51)

Apart from the fact that non haemolytic streptococci occurred more frequently in healthy ears than in affected ears, it was noticed that in primary cultures of otitic material the streptococci were rarely present in large numbers and that pure cultures were obtained in only 15 (3 per cent) of the 523 affected ears.

The detailed study of the non haemolytic streptococci was restricted to 79 strains of which 29 were alpha haemolytic and 50 were non haemolytic.

TABLE 37

The classification of non haemolytic streptococci from the external ears of normal and affected dogs

	Affected ears				Healthy ears	
	Alpha haemolytic strains	Non haemolytic strains	Total all non haemolytic strains		All non haemolytic strains	
			No.	%	No.	%
Group C.	1	-	1	(1)	-	-
Group L.	2	-	2	(3)	-	-
Strep. faecalis	6	21	27	(34)	13	(52)
Strep. liquefaciens	5	21	26	(33)	-	-
Strep. bovis	9	2	11	(14)	3	(12)
Strep. equinus	4	1	5	(6)	2	(8)
Strep. salivarius	1	5	6	(8)	5	(20)
Strep. glycerinae	1	-	1	(1)	-	-
Strep. mitis	-	-	-	-	2	(8)
Total	29	50	79		25	

These results show Strep. salivarius to be more common in the external ears of healthy dogs than of dogs affected with otitis.

If it be accepted that the streptococcal species faecalis, liquefaciens, bovis and equinus are common in the animal intestine, it will be observed that 72 and 87 per cent respectively of the non haemolytic streptococci from healthy and infected external ears were of faecal origin. Although the difference of 15 per cent is probably of little significance, it would appear that Strep. liquefaciens is a common inhabitant of infected ears (33) per cent but not of healthy ears.

Diphtheroid bacilli:

It has already been shown that Gram positive rods resembling Corynebacterium species were present in the ears, nose and tonsils of normal dogs. Similar organisms were isolated from 89 (17 per cent) of the 523 infected ears, although they were rarely present in appreciable numbers. This, and the fact that pure cultures of diphtheroid bacilli were obtained from only 8 (2 per cent) of the external ears of dogs affected with otitis suggested that their presence was of little importance.

The results in Table 38 show their frequency of occurrence in healthy and affected tissues.

TABLE 38

The incidence of diphtheroid bacilli in healthy and affected tissues

	Affected dogs	Healthy dogs			
	External ears	External ears	Middle ears	Nose	Tonsils
No. of swabs	523	70	50	35	35
No. of strains	89	11	1	6	8
Per cent positive	(17)	(16)	(2)	(17)	(23)

The findings in Table 38 would appear to support the suggestion that the presence of diphtheroids, in cases of otitis, is not of pathogenic significance. It will be seen that they occurred just as often in healthy tissues (with the exception of the middle ears) as in the external ears of affected animals.

The biochemical characters of 80 strains from infected ears were examined in some detail.

The "diphtheroids" from normal tissues were divided into the two species, Corynebacterium hoffmanni and Corynebacterium xerosis, according to their morphological characteristics and their ability to ferment carbohydrate media. C. hoffmanni was identified as a uniformly staining club-shaped rod which did not ferment sugars whereas C. xerosis was a more pleomorphic rod which was more active in carbohydrate media. Their incidence in otitic material was as shown in Table 39.

TABLE 39

The incidence of diphtheroid bacilli in healthy and affected animals

Number of strains examined		Number (percentage) of strains	
		C. hoffmanni	C. xerosis
Otitic material			
External ears	80	53 (66.3)	27 (33.8)
Normal material			
External ears	12	8	4
Middle ears	1	1	-
Nose	6	3	3
Tonsils	10	9	1
Total	29	21 (72.4)	8 (28.6)

These results show that the diphtheroid bacilli of canine origin included two important species, viz: C. hoffmanni and C. xerosis, the former being almost twice as common as the latter. Both species were equally common in the external ears, whether or not an otitis was present.

The subtilis group:

The aerobic sporing bacilli were identified according to the classifications of Gibson (1944), Gordon et al (1952) and Knight et al (1950). Altogether 62 (12 per cent) of the 523 affected ears showed the presence of "Subtilis-like" bacilli

bacilli which may be compared with those of healthy tissues as follows:-

TABLE 40

The incidence of sporing bacilli in healthy and affected tissues

	Affected dogs	Normal dogs			
	External ears	External ears	Middle ears	Nose	Tonsils
No. positive	62	22	3	14	8
Per cent positive	(12)	(31)	(6)	(40)	(23)

The fact that aerobic sporing bacilli were recovered from 31 per cent of normal ears, as against 12 per cent of affected ears confirmed the opinion that their presence in otitic material was of little importance. It will also be shown that the Subtilis group consisted of a number of different species, all of which are common soil parasites.

In contrast to the 10 species from normal tissues, only 6 species were identified in cultures prepared from the otitic material.

TABLE 41

The species of aerobic sporing bacilli in healthy and affected tissues

Sites examined	Number of strains isolated	B. subtilis	B. cereus	B. mycoides	B. megatherium	B. pumilus (mesentericus)	B. licheniformis	Other species
Affected ears	62	23	9	11	6	3	10	-
Healthy -								
External ears	30	8	2	4	3	2	2	9
Middle ears	3	1	1	-	-	-	-	1
Nose	15	5	2	4	2	-	-	2
Tonsil	8	4	-	2	2	-	-	-
Normal total	56	18	5	10	7	2	2	12

Although it was anticipated that the majority of the strains would be typical B. subtilis, the high incidence of B. licheniformis was unexpected. The B. cereus group was exceptional in that B. cereus was recovered in pure culture on three separate occasions from the same ear, over a period of five months.

Pasteurella:

It has already been shown that small Gram negative, bipolar

bipolar staining rods which resembled Pasteurella species were frequently present in the tonsils of healthy dogs. Although only one of the strains was identified as a typical Pasteurella septica, it was thought possible that these other organisms, hereafter referred to as 'Pasteurella species', might spread by way of the eustachian tubes to the middle and outer ears, giving rise to an otitis.

During the period of this survey cases of otitis in cats have been observed in this School where the causal organism was considered to be Past. septica. A similar organism was also isolated from skin lesions in cats during the same period. It is surprising, therefore, to find that out of 523 infected canine ears, Past. septica was recovered on only 3 (0.6 per cent) occasions. Moreover, in none of these ears was there evidence of the other Pasteurella species which were present in 31 per cent of healthy tonsils. Smith (1955) noted organisms which he called Past. septica in 11 (10 per cent) nose and 59 (54 per cent) tonsil swabs of 111 healthy dogs.

TABLE 42

The incidence of *Pasteurella* species in healthy dogs and in the ears of dogs affected with otitis

Number of sites examined		*Pasteurella* species Number positive	%	*Pasteurella septica* Number positive	%
Infected (external ears)	523	-	-	3	0.6
Healthy (external ears)	70	-	-	-	-
Healthy (middle ears)	50	2	(4)	1	(2)
Healthy nares	35	5	(14)	-	-
Healthy tonsils	35	11	(31)	1	(3)

These results show that *Past. septica*, and the so-called *Pasteurella* species are probably of little importance so far as external otitis in dogs is concerned. This latter group of organisms are un-named, as they appeared to be distinct from other previously described *Pasteurella* species.

The 5 strains of *Past. septica*, 3 of which were from infected ears and 2 from healthy dogs, were submitted to the usual physiological and biochemical tests with the following results. (The '*Pasteurella* species' from healthy dogs have already been considered in Part I).

TABLE 43

The characteristics of 5 strains of Past. septica from healthy and affected dogs

	Number of strains			Number of strains	
	+	-		+	-
Motility	-	5	Acid but not gas from:-		
Gelatin	-	5	Glucose	5	-
Solid serum	-	5	Lactose	-	5
			Maltose	-	5
Litmus milk	-	5	Mannitol	5	-
Methylene blue	-	5	Dulcitol	-	5
V.P.	-	5	Inositol	-	5
M.R.	-	5	Saccharose	5	-
Catalase	3	2	Sorbitol	5	-
Nitrates	5	-	Salicin	-	5
Urea	-	5	Raffinose	-	5
Indole	5	-	Arabinose	1	4
H_2S	5	-	Trehalose	5	-
Growth on			Xylose	3	2
MacConkey	-	5	Dextrin	-	5
Lethal to			Inulin	-	5
mice	5	-	Rhamnose	-	5
			Glycerol	-	5
			Laevulose	5	-
			Galactose	5	-
			Mannose	5	-

The strains of Past. septica from dogs differed from the other Pasteurella species in that they consistently fermented mannitol and mannose but never maltose, inositol or dextrin. The former group was also distinguished by their marked ability to reduce nitrates to nitrites and the fact that they were always lethal to mice.

The anaerobic spore-forming bacilli:

Although occasional colonies of anaerobic sporing bacilli were frequently present on the surface of primary culture plates, the organisms were rarely seen in stained smears of the otitic material. Moreover, as sporing anaerobes were commoner in healthy than in infected external ears, this suggested that they were of little importance in canine otitis.

The present investigation was confined to a study of the external ears of the 35 clinically healthy dogs and of 290 consecutive cases of otitis externa. The anaerobic bacilli present were classified, for convenience, in the following groups:-

Group 1: Bacilli resembling Clostridium welchii

Group 2: Small, weakly Gram positive bacilli, with terminal oval or spherical spores.

Group 3: Bacilli with large central, eccentric or subterminal oval spores.

Anaerobic spore-forming bacilli were isolated from 31 (44 per cent) of the healthy ears and from 123 (42 per cent) of the infected ears, and were provisionally identified as shown in Table 44.

TABLE 44

The anaerobic aporing bacilli isolated from healthy and infected external ears

Healthy ears examined - 70

Infected ears examined - 290

Ears infected with anaerobes		Provisional Identification					Total No. of strains isolated
		Group 1			Group 2	Group 3	
		B.	a.	-.			
Healthy ears	31	15	7	3	3	7	35
	(44)	(21)	(10)	(4)	(4)	(10)	
Otitic ears	123	43	19	10	20	46	138
	(42)	(15)	(7)	(3)	(7)	(16)	

NOTE: B. = Beta haemolytic
a. = alpha haemolytic
-. = non haemolytic

The figures in brackets are expressed as a percentage of the total number of ears examined.

These results show that Group 1 (Cl. welchii) species were commoner in healthy ears (36 per cent) than in otitic material (25 per cent), whereas the incidence of other anaerobic bacilli was greater in the infected ears. Of the 97 strains resembling Cl. welchii only 58 gave the typical beta-type haemolysis on horse blood agar plates.

All the anaerobic sporing bacilli were identified according to the classifications of Bergey (1948).

Clostridium welchii:

Although the stormy clot reaction cannot be regarded as a specific indicator of Cl. welchii, for the reaction may be given by Cl. aerofoetidum, Cl. multifermentans and Cl. butyricum (Spray, 1936), positive identification required the development of a stormy clot and the presence of Gram positive non-sporing bacilli in films of the milk (Willis, 1957), provided that the strain was morphologically typical and gave characteristic cultural and fermentation reactions.

Morphology:

All strains presented the typical morphological appearance of Cl. welchii being non-motile stout rods with parallel sides and truncated or slightly rounded ends and which rarely formed spores in cultures.

Cultural characters:

Horse blood-agar: The chief variation in cultural characters was the colonial appearance on 5 per cent horse blood-agar. After 48 hours at 37°C., low convex colonies, 2 - 4 mm. in diameter were obtained that were smooth, semi-translucent and with an entire edge; although many were umbonate and showed radial striations and a crenated edge. The degree of haemolysis varied from strain to strain, a zone of beta haemolysis being

being produced around the colony in most strains, while others were weak or non haemolytic, even after 24 hours in the dark at room temperature.

Gelatin and solidified serum media: All the welchii strains from dogs were typical in that they liquefied gelatin promptly but failed to hydrolyse inspissated bovine serum slopes.

Cooked meat media: All grew well in meat broth producing a slight turbidity of the supernatant fluid. Most strains formed variable amounts of gas, turned the meat pink and gave off a faintly sour odour.

Biochemical: The welchii strains were indole, catalase and V.P. negative and failed to reduce methylene blue. They were also M.R. positive and formed a typical stormy clot, within 48 hours, in litmus milk media.

Sugar fermentation: There was little variation in the sugar fermentation reactions, most strains producing acid and gas in glucose, lactose, maltose and sucrose. Only 18 (28 per cent) fermented salicin whereas none attacked mannitol.

Typing of cultures:

In her report on the occurrence of Cl. welchii in normal animals, Borthwick (1937) noted the presence of Cl. welchii types A. and D. in dogs. Although Taylor and Gordon (1940) found that all their 110 cultures, from the intestinal contents of 17 out of 21 dogs, were type A., strains of type D were isolated from the intestines of only one sheep, one bovine and one domesticated

domesticated rabbit. They also examined 196 cultures from 43 soil samples and found that 189 were either of type A., or were non toxic, the remainder being type D.

Because of the variation in the colonial characters of the Cl. welchii of canine origin, an experiment was carried out to determine which toxins, if any, they might produce. The methods used were almost identical to those of Taylor and Gordon (1940) except that each strain was subcultivated twice daily for 4 days, in meat broth, and finally overnight at 37°C. The antitoxins used were types A. and B. except in the case of a number of strains which apparently failed to form toxin, and which were re-examined for type D. toxins by incubating the meat broth cultures for 4 days before use; to allow maximum development of type D. toxin. In this instance type A. and type D. antitoxins were used.

Of the 30 strains examined, 20 were beta, 5 alpha and 5 non haemolytic; they were otherwise unselected.

TABLE 45

The types of Cl. welchii from the external ears of dogs

Degree of haemolysis	No. of strains examined	No. of type A. cultures	No. of cultures apparently non-toxic
Beta	20	16	4+
Alpha	5	5	-
Non haemolytic	5	-	5+
Total	30	21	9

NOTE: '+' = These cultures also failed to show type D. toxin

These results show that most welchii strains of canine origin, which exert an haemolytic effect on horse red cells, are of type A. Four haemolytic strains apparently failed to produce detectable amounts of toxin, or were non-toxigenic, as were the 5 non haemolytic strains.

Approximately 13 per cent of the welchii strains from dogs were non haemolytic and although non haemolytic heat resistant strains have been reported (Hobbs, Smith, Oakley, Warrack and Cruickshank, 1953) from 18.4 per cent and 1.7 per cent respectively of the faeces of pigs and cattle, none of the non haemolytic strains from dogs' ears resisted gentle boiling for one hour. It is of interest that Willis (1957) examined 50 strains of

of Cl. welchii from a water supply and found that several were not only non haemolytic and non-heat resistant but were only feebly toxigenic.

TABLE 46

The characters of 65 strains of Cl. welchii from external ears

Character	No.		No.
Colony - rough	48	Cooked meat medium	
- smooth	17	- reddened	65
		- gas produced	61
Haemolysis on horse blood			
- beta	40	Indole	0
- alpha	13		
- none	12	Catalase	0
Litmus milk, stormy clot		Methyl Red	63
- marked	58	Voges-Proskauer	0
- weak	7		
		Fermentation of -	
Methylene blue reduced	0		
		Glucose	65
Gelatin liquefied	65	Lactose	65
		Maltose	65
Serum digested	0	Mannitol	0
		Sucrose	63
		Salicin	18

Number of strains giving a positive reaction

Although Cl. welchii was thought to be of little importance in canine otitis, the number of strains isolated was greater than the combined total of the other sporing anaerobes the figures being, for 'otitis strains' 25 per cent and 23 per cent

per cent respectively.

The strains in Group 2 of the provisional classification included the weakly Gram positive bacilli with terminal oval, or spherical spores. Of the 23 strains isolated 20 were from infected external ears. Three species were identified namely Cl. cochlearium (8 strains), Cl. tetanomorphum (5 strains) and Cl. tertium (10 strains).

Cl. cochlearium: This was recognised as a long, slender, weakly Gram-positive rod forming terminal oval spores, twice the width of the bacillus. On the surface of horse blood-agar plates a small, non haemolytic, translucent colony was formed, the edge of which was regular or very slightly crenated, although on prolonged incubation it tended to form tiny lateral tufts of growth. None of the strains fermented sugars, formed indole or liquefied gelatin or solidified bovine serum. There was no reaction in litmus and methylene blue media and the V.P. , M.R., catalase and nitrate reactions were also negative.

Cl. tetanomorphum: The colonies of Cl. tetanomorphum were almost transparent and were flatter and more irregular than those of Cl. cochlearium. These organisms were also very similar morphologically, although the spores of Cl. tetanomorphum were spherical rather than oval. A number of strains formed indole but litmus milk, gelatin and serum media were unaffected. They all produced acid and gas in glucose and maltose but not in

in lactose, mannitol or sucrose.

Cl. tertium: The terminal spores of Cl. tertium were oval or occasionally distinctly spherical. Unlike the other two species in this group the colonies on blood agar were small and regular and usually surrounded by a faint but unmistakable zone of haemolysis. Acid was formed in litmus milk and gas in meat broth.

The reactions given by the anaerobic terminal sporing bacilli are summarised in the following Table:-

TABLE 47

The characters of the terminal sporing anaerobes

	Number of strains giving a positive reaction		
	Cl. cochlearium	Cl. tetanomorphum	Cl. tertium
Haemolysis	0	0	10
Acid in litmus milk	0	0	10
Reduction of methylene blue	0	0	0
Liq. of Gelatin	0	0	0
Liq. of inspissated bovine serum	0	0	0
Cooked meat medium			
- reddened	8	5	10
- gas formed	0	0	10
Indole	0	2	4
Motility	8	5	10
Acid and gas from -			
Glucose	0	5	10
Lactose	0	0	8
Maltose	0	5	10
Mannitol	0	0	4
Sucrose	0	0	10
Salicin	0	0	6
Laevulose	0	4	9
Galactose	0	5	10
Glycerol	0	4	0
Number of strains examined	8	5	10

The 53 anaerobic strains with large central, or sub-terminal oval spores, (provisional Group 3) were identified as follows:-

Cl. butyricum	-	14 strains
Cl. multifermentans	-	7 strains
Cl. sporogenes	-	6 strains
Cl. bifermentans	-	11 strains
Cl. aerofoetidum	-	4 strains
Unidentified Clostridium spp.	-	11 strains

Cl. butyricum: Most butyricum strains formed greyish or creamy white irregular, non haemolytic, butyrous and easily emulsifiable colonies on horse blood agar. They all fermented a variety of sugars, failed to liquefy gelatin and solified bovine serum, were indole and nitrate negative, did not digest cooked meat medium but produced acid and coagulum in litmus milk.

Cl. multifermentans: The characters of most strains were similar to those of Cl. butyricum except that most multifermentans strains reduced nitrates to nitrites, were variably haemolytic, did not ferment mannitol and formed low convex, regular surface colonies.

Cl. sporogenes: The colonial characters of Cl. sporogenes differed from the above in that they were small, irregular, often greyish-white, usually fimbriate and haemolytic. Meat broth

broth cultures were digested, blackened and produced a foul odour, with gas formation. All strains completely digested gelatin and solidified serum media, while of the three cultures tested for pathogenicity to guinea pigs all proved negative.

Cl. aerofoetidum: This species was identified as forming small, transparent, non haemolytic colonies with a characteristic bluish tint. Acid was usually formed in litmus milk and a pink colouration was produced in cooked meat medium, which was slowly digested. All strains liquefied both gelatin and serum media.

Cl. bifermentans: Unlike the above strains, the bifermentans bacillus was not distinctly swollen at sporulation. The colonies, on horse blood agar were small, circular or irregular, fimbriate and occasionally haemolytic. Gelatin and inspissated serum media were liquefied, indole was formed and cooked meat medium was blackened and digested, with a foul odour. Most strains produced acid and gas in glucose, maltose and salicin.

A summary of the characters of these central or sub-terminal oval sporing anaerobic bacilli is shown in the following Table.

TABLE 48

The characters of the central and subterminal oval spore-forming anaerobic bacilli

Character	Species and number of strains giving a positive reaction				
	Cl. butyricum	Cl. multifermentans	Cl. sporogenes	Cl. aerofoetidum	Cl. bifermentans
Bacillus swollen at sporulation	14	7	6	4	0
Haemolysis	0	7+	6	0	3
Cooked meat medium -					
Gas	14	7	6	2+	11
Foul odour	0	0	6	4+	11
Blackening	0	0	6	4+	11
Liquefaction of gelatin	0	0	6	4	11
Digestion of serum	0	0	6	4	11
Acid formed in					
Litmus milk	13	7	6	4	11
Nitrates reduced	0	7	0	0	0
Indole	0	0	0	3+	11
Fermentation of -					
Glucose	14	7	6	4	11
Lactose	14	7	0	4	0
Maltose	14	7	6	4	11
Mannitol	9	0	0	0	0
Sucrose	14	7	0	0	0
Salicin	12	7	5	3	8
Laevulose	14	7	5	4	10
Galactose	14	7	5	4	10
Glycerol	5	7	0	0	8
Number of strains examined	14	7	6	4	11

NOTE: '+' = a weak or irregular reaction

Fungi and Yeasts:

The mycological examinations were confined to the study of 400 affected external ears, the presence of a particular fungus being recorded only if it were seen in film preparations and in primary cultures, or if it grew in both the original and duplicate cultures.

TABLE 49

The incidence of yeasts and fungi in the external ears of normal and affected dogs

Number of healthy ears examined - 70

Number of infected ears examined - 400

Genus.	Healthy ears positive		Infected ears positive	
	No.	%	No.	%
Aspergillus	4	(6)	13	(3)
Penicillium	3	(4)	12	(3)
Mucor	-	-	3	(0.8)
Rhizopus	3	(4)	6	(1.5)
Absidia	2	(3)	4	(1)
Cladosporium	1	(1)	8	(2)
Geotrichum	1	(1)	3	(0.8)
Botrytis	-	-	1	(0.3)
Candida	3	(4)	19	(5)
Rhodotorula	-	-	5	(1)
Pityrosporum	25	(36)	177	(44)

Altogether 251 strains of fungi, yeasts and yeast-like organisms were isolated from 241 (60 per cent) of the 400 affected ears.

With the exception of Pityrosporum species, which were present in 44 per cent of infected ears, neither the yeasts nor

nor the true fungi are of special importance as is shown by the fact that the most prevalent fungus, Aspergillus species, was present in only 3 per cent of cases.

The various fungal species were identified by their growth characteristics on selected media, slide culture techniques being invaluable in this respect. The generic name only was used to describe a number of species which were not fully identified, e.g. Penicillium species.

In every case, where a positive diagnosis was obtained, each strain was checked against a known strain from a collection kindly supplied by Dr. G.C. Ainsworth. The classification of the 50 fungal strains from affected ears is included in Table 50.

TABLE 50

The species of fungi isolated from infected ears

	Species	No. of strains
Phycomycetes:	Absidia ramosa	3
	A. corymbifera	1
	Mucor racemosus	3
	Rhizopus species	2
	R. nigricans	4
Fungi Imperfecti		
	Aspergillus fumigatus	7
	A. niger	2
	A. glaucus series	1
	A. versicolour	1
	Aspergillus species	2
	Botrytis cinerea	1
	Candida albicans	11
	C. tropicalis	5
	C. krusei	2
	C. parapsilosis	1
	Cladosporium herbarum	8
	Geotrichum candidum	3
	Penicillium expansum	4
	P. nidulans	2
	P. spinulosum	1
	Penicillium species	5
	Pityrosporum species	177
Rhodotorula sp.	Rhodotorula species	5

Candida species:

Woods (1951), Moore (1951) and Huppert, McPherson and Cozin (1952) have demonstrated the importance of yeasts as a cause of certain side effects during treatment with antibiotics, and Duncan (1945) has reported Candida albicans infection in 3 cases of acute mycotic otitis in man.

In this survey Candida species were isolated from 19 (5 per cent) of the infected outer ears. These included 11 strains of C. albicans, 5 of C. tropicalis, 2 of C. krusei, and 1 of C. parapsilosis.

The diagnosis of the genus Candida was based on the recommendations of Lodder and Kreger-van Rij (1952). All members of this group showed large oval or rounded cells which reproduced by multilateral budding. The development of a well-defined pseudomycelium was a fairly constant feature, although chlamydospore formation was seen only in cultures of C. albicans. Most strains fermented glucose and a number of other sugars, while in other fluid media a surface pellicle and ring formation was frequently present. Strains of C. albicans which produced white to ivory coloured creamy colonies on solid media with a well-developed pseudo-mycelium were distinguished from other members of the genus in that they produced both acid and gas in glucose, galactose and maltose, but acid only in saccharose. On corn-meal agar most strains showed chlamydospore formation, although carrot-plug cultures never formed asci. The lethal

lethal effect of many of the strains was demonstrated by the intravenous inoculation of 0.2 ml. of an overnight broth culture in rabbits and mice which is in marked contrast to the strains of C. tropicalis. They were never pathogenic to laboratory animals and were able to produce both acid and gas in saccharose. Neither saccharose nor maltose was fermented by the single strain of C. parapsilosis. C. krusei, on the other hand, was distinguished by the fact that it fermented glucose only, and that it frequently showed elongated cylindrical cells in 3 day old cultures at 25°C.

Although the incidence rate of 5 per cent is probably not significant, it was found that at least three of the dogs affected with chronic otitis developed a mycotic infection as the result of antibiotic therapy and, in each case, the organism concerned was C. tropicalis.

The part played by Pityrosporum species is not obvious. This organism appears to be closely related to the Pityrosporum ovale of man which, by invading the epidermis, is said to cause a multiplication of the cells with a consequent scaling of the horny layer. P. ovale has frequently been described as the cause of seborrhoea, dandruff and a number of other skin conditions.

Although the canine Pityrosporum species differ from the human strains in that they do not require the addition to the media of oily substances such as oleic acid, it is nevertheless possible that they too may give rise to an inflammatory reaction

reaction in the external acoustic meatus. Whilst the figure of 44 per cent would appear to support this theory, it will be noticed that Pityrosporum species were isolated from not less than 36 per cent of healthy ears. It may be recalled that these figures are very similar to those obtained for staphylococci from healthy and infected ears, and it is probably equally true that the Pityrosporum species are normal commensals of the ear which are capable of rapid multiplication under certain favourable circumstances.

In an attempt to confirm this, cases of otitis in which Pityrosporum yeasts were the only organisms present were treated with a variety of substances, the antiseptic substances being those to which the Pityrosporum strain was sensitive in vitro. The results of this experiment were disappointing in that the clinical picture was unchanged in most cases long after the yeasts were eliminated.

A number of strains from infected material were also inoculated into white mice by various routes, including skin scarification, but at no time was there evidence of tissue reaction.

PART II - SUMMARY:

The flora of 523 ears of 363 dogs affected with external otitis has been considered and the findings compared with those of the external and middle ears, the anterior nares and the tonsils of 35 healthy dogs. The dogs were chosen at random and no attempt was made to differentiate the different clinical types of otitis. Patients with a previous history of otitis were not included in this part of the survey and all the swabs were taken before treatment began.

It is generally believed that some breeds are more prone to ear canker than others. The truth of this statement is well illustrated by the fact that one in three (37 per cent) of the affected dogs were Spaniels. Unfortunately, the number of Spaniels in the dog population of this country is not known but in the first month of this year (1957) 63 (10 per cent) of the 618 dogs which passed through the "out-patient" Department of this School were Spaniels (Gregor, 1957).

The cultural examinations of otitic material showed that the more frequently occurring micro-organisms could be conveniently classified into eight or nine groups, the most important of which were Pseudomonas, Proteus, Staphylococcus and Pityrosporum.

Although staphylococci and Pityrosporum species were present in 61 and 44 per cent respectively of affected ears, they were almost as prevalent in normal ears, the carrier rates for which

which are respectively 54 and 36 per cent. As nearly half (46 per cent) of the staphylococci in normal ears were coagulase producers, their presence may be of aetiological significance by virtue of the fact that they may reach the underlying tissues of the external meatus by way of a small abrasion or break in the continuity of the stratified squamous epithelium. This desquamation of the surface cells may be caused by excessive moisture, such as perspiration or by irritation of the lining membrane by foreign bodies or ectoparasites. It will also be shown that the great majority of coagulase positive staphylococci of canine origin produce dermonecrotic toxin.

Similarly, Pityrosporum species which are commonly associated with dandruff and other skin conditions may reach the subcutis of the external meatus giving rise to the typical early acute manifestations of otitis. On the other hand, large numbers of staphylococci and Pityrosporum species in infected material may be due to the fact that these commensals are capable of rapid multiplication under favourable conditions.

Whilst Proteus and Pseudomonas species were not present in the ears of healthy dogs, 16 and 13 per cent respectively of the affected ears were so infected. Not only is this difference significant but, as will be shown in Part III, the number of ears infected with one or both of these organisms increased during and after treatment. As Proteus and Pseudomonas species were not normally present in dogs' ears, their presence in

in otitic material must be the result of infection from an external source. It was shown, by means of the Dienes' phenomenon that, in the great majority of cases of bilateral otitis, due to Pr. mirabilis, both strains were antigenically similar which suggested that the source of infection may be from one ear to the other or by contamination from a common source. A study of the rectal strains from the same dogs suggested the intestine as the most likely source of infection (Part VI). The fact that 40.5 per cent of Pr. mirabilis strains were antigenically similar implied either that a particular strain is predominantly pathogenic to dogs, or that the unknown reservoir of infection consists mainly of strains of one particular type.

The presence of coliform organisms in 6 per cent of healthy ears but in only 14 per cent of the infected material may, nevertheless, be of importance as 95 per cent of the E. coli strains were probably of faecal origin. An important feature of the Pseudomonas group was that all strains were finally identified as Ps. aeruginosa, although only 52 per cent produced typical pyocyanin pigment.

Of the 82 Proteus strains from infected ears, 79 were Pr. mirabilis, 2 were Pr. vulgaris and the remaining strain was an atypical Pr. morganii. All but 12 of the 79 Pr. mirabilis strains fell into 14 distinct serological groups of which the largest contained no fewer than 32 (40 per cent) strains.

The majority of the staphylococci were coagulase positive,

positive, actively proteolytic, non-pigment producing species which rarely formed alpha-type toxin and attacked mannitol irregularly. They appeared to differ, in a number of respects, from the typical Staph. aureus of human and bovine origin.

The haemolytic streptococci which were present in 18 per cent of the affected ears were classified by Lancefield's techniques into six serological groups in the following order of frequency: Groups G. L. C. D. M. and B. Of these, group G. strains were by far the commonest.

The other bacterial species would appear to be of little practical importance and, in the discussion of the Pasteurella group, the low incidence of Past. septica was stressed.

The incidence of anaerobic sporing bacilli in healthy and infected external ears was similar, viz. 44 and 42 per cent respectively. The commonest species was Cl. welchii, almost 62 per cent of which were beta haemolytic. Of 30 welchii strains examined, 16 beta, all 5 alpha but none of the 5 non haemolytic strains produced type A. toxin.

Also common, in the external ears of healthy and affected dogs, were the anaerobic, central or subterminal, oval sporing bacilli (10 and 16 per cent respectively) and the terminal sporing anaerobes (4 and 7 per cent respectively).

With the exception of Pityrosporum species, fungi were rarely recovered from otitic material, the most important potential pathogen, Aspergillus fumigatus, being recovered from

from only 3 per cent of infected ears.

The correlation of the bacterial findings to the nature of the discharge showed that, while there was some connection between the more profuse type of discharge and the Gram negative bacteria, the other bacterial species were unrelated to the quantity of exudate. However, if the colour of the material was also taken into account, it would appear possible for the Pseudomonas and Proteus infected ears which produce a copious, evil-smelling, wet, pale yellow discharge to be differentiated from the Pityrosporum and staphylococcal types with their drier, reddish-brown or chocolate-brown coloured exudate.

PART III

The flora of the ears of dogs suffering from otitis, which failed to respond to treatment

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This section will be considered in two parts as follows:-

PART III.a: The external ears of 100 consecutive cases of otitis which were examined before treatment and at various intervals thereafter and which failed to respond to treatment within three weeks.

PART III.b: The ears, anterior nares, tonsils and recta of 23 dogs which were suffering from chronic otitis, when first examined.

Part III a.

Details of the age, sex and breed of the 100 dogs which did not respond promptly to treatment (Table 51) show that Spaniels, Labrador retrievers and certain types of Terrier are more frequently affected with external otitis than are other dogs. By comparing these findings, with those of Part II (summarised in Table 52), it will be seen that the number of Spaniels that did not readily respond to treatment was increased by about 9 per cent. It was also evident that, apart from the Spaniel, there was little or no correlation between the conformation of the ear and a predisposition to otitis.

TABLE 51

The age, sex and breed of dogs that failed to respond to treatment

Number of dogs examined	100		Bilateral otitis	51		
Number of ears examined	151		Unilateral otitis	49		
			Left ears	31		
			Right ears	18		

	Male	Female	Total		Male	Female	Total
+ Alsatian	2	2	4	"Terrier Types"			
+ Collie	2	1	3	+ Scotch Terrier	5	1	6
+ Corgi	-	1	1	+ Fox Terrier	1	-	1
+ Chow	1	-	1	+ West Highland Terrier	2	1	3
Spaniel	28	14	42	+ Bull Terrier	2	-	2
Labrador	8	2	10	Border Terrier	5	1	6
Boxer	2	-	2	Airedale	1	-	1
Poodle	2	1	3	Mongrels			
Dachshund	1	2	3	"Cross" Terrier	5	5	10
Pyrenean Mountain	1	-	1				
Pekingese	-	1	1	Total	21	8	29
Total	47	24	71	GRAND TOTAL	68	32	100

NOTE: '+' = Dogs with prick or semi-prick ears

The average age was 5 years and 7 months

TABLE 52

Breed susceptibilities to otitis

Percentage number of dogs examined

	Before treatment only (Survey - Part II)	Before and during treatment (Survey - Part III.a.)	Percentage increase
+ Alsatian	6	4	-
+ Collie	7	3	-
Spaniel	34	42	8
Labrador	9	10	1
Boxer	3	2	-
Poodle	3	3	-
Dachshund	2	3	1
+ Scotch Terrier	5	6	1
+ Cairn Terrier	3	-	-
+ Fox Terrier	3	1	-
+ West Highland	3	3	-
+ Bull Terrier	1	2	1
Border Terrier	2	6	4
Airedale	1	1	-
"Cross" terrier	8	10	2

Note: (+) = dogs with 'prick' or 'semi-prick' ears.

The micro-organisms present in the 151 affected ears were classified, as before, into the following groups:- Pseudomonas, Proteus, the coliform group, haemolytic streptococci, non haemolytic streptococci, staphylococci, 'diphtheroids', the Subtilis group, Pityrosporum and other yeasts.

The flora of the infected ears that resisted treatment will be discussed in two parts, the first of which will be of the ears just before treatment began. The second part, while ignoring these results, will only deal with the organisms which were present in the ears on subsequent examinations during the course of treatment. By comparing the two sets of results (as in Table 53) it may be possible to express an opinion as to the importance of any particular species. The results of the earlier survey will also be included.

TABLE 53

The micro-organisms in external otitis, before and during treatment

	Otitis material		
	Survey - Part II 523 ears	Survey - Part III 151 ears	
	Before treatment (% infected)	Before treatment (% infected)	After treatment (% infected)
Pseudomonas	13	15	19
Proteus	16	17	22
Coliforms	14	9	13
Haemolytic streptococci	18	15	15
Non haemolytic streptococci	25	18	13
Staphylococci	61	49	47
Diphtheroids	17	8	13
Subtilis group	12	11	9
Pityrosporum	44	19	36
Other yeasts	5	1	3

If the findings from the "pre-treatment" ears of Parts II and III are compared, it will be noticed that only Pseudomonas and Proteus species were more frequent in the ears which later proved to be resistant to treatment. The lower incidence of all other species suggested that their presence in this type of infection was of no great significance.

Several unexpected results were obtained when the findings of the survey in Part III.a. were compared with each other. During the course of treatment, instead of a fall in the incidence of the different species, the number of ears infected with Ps. aeruginosa, Pr. mirabilis and coliforms actually increased. That this was not due to faulty treatment was shown by the fact that many of the ears so infected, at the time of the first examination, cleared up quickly and completely. Instead it was found that the increase occurred during treatment, or shortly thereafter. This is illustrated in the following Table.

TABLE 54

Pseudomonas and Proteus species in affected ears before and during treatment

	Pseudomonas aeruginosa		Proteus mirabilis	
	Dogs (100)	Ears (151)	Dogs (100)	Ears (151)
Number infected -				
before treatment	18	23	18	26
after treatment started	8	10	12	14
Total	26	33	30	40

These results show that of 100 dogs suffering from an otitis which resisted treatment, 18 were infected with Ps. aeruginosa and the same number with Pr. mirabilis, at the time when the dog was first examined, whereas 8 animals acquired a Pseudomonas infection and 12 a Proteus infection at some point after treatment began. It would also appear that 31 per cent and 40 per cent of all the dogs showing Pseudomonas or Proteus species respectively, in the external ears, became infected after the start of treatment. This suggested that the initial lesion in the ear was not of bacterial origin and that the organisms were present as the result of secondary infection from an external source.

A similar but less spectacular increase occurred with the coliforms, although the frequency of the other bacterial species tended to fall.

The fact that the yeasts and, in particular, Pityrosporum species became more numerous during treatment was probably due to the effect of antibiotics on the yeast-cell's metabolism, or indirectly to their more rapid multiplication in the presence of a reduced bacterial population in treated ears.

During the period of this survey most of the dogs were treated with proprietary preparations incorporating both penicillin and streptomycin.

Part III b

Chronic otitis of the external and middle ears

Material was examined from the ears, anterior nares, tonsils and recta of 23 dogs, all of which were suffering, when first examined, from a chronic otitis of many months' standing. In the case of 17 dogs, that were destroyed for humane reasons, material was also obtained from the middle ears.

TABLE 55

The number of sites examined of 23 dogs suffering from chronic otitis

Site	No. examined
External ears	46
Middle ears	34+
Anterior nares	23
Tonsils	23
Rectum	22

'+' = Material obtained from 17 cadavers

This investigation was carried out in an attempt to trace the probable source and spread of infection in cases of canine otitis.

The incidence of the more important bacterial species in a) unselected cases of otitis, b) in infected ears which failed to respond to treatment and c) in cases of chronic otitis (Parts II, IIIa and IIIb respectively) are compared in Table 56.

TABLE 56

The flora of chronically infected ears - compared with the findings of the previous surveys

	Survey Part II 523 ears Before treatment (% infected)	Survey Part III.a 151 ears Before (% infected)	Survey Part III.a 151 ears After Treatment (% infected)	Survey Part III.b 46 ears Chronic otitis (% infected)
Pseudomonas	13	15	19	48
Proteus	16	17	22	39
Coliforms	14	9	13	24
Haemolytic streptococci	18	15	15	28
Non haemolytic streptococci	25	18	13	52
Staphylococci	61	49	47	57

The results in Table 56 show that the more chronic the otitis the greater is the incidence of streptococci and the Gram negative species, Pseudomonas, Proteus and the coliforms.

The much increased frequency of Pseudomonas and Proteus, in cases of chronic otitis, is probably responsible for the purulent evil-smelling exudate and the excessive thickening of the epithelium which is, invariably, a diagnostic feature of the more resistant types of otitis.

The fact that coliforms and haemolytic and non haemolytic streptococci are also commoner in chronically infected ears is probably due to the lesion becoming infected from an external source or by auto-infection from the intestines. On the other hand, the relative stability of the staphylococcal population suggests that they are normally present in the healthy meatus.

By comparing the findings of the other sites with those of the chronically infected external ears, it was possible to identify the most likely source of infection within the animal body. In Table 57, which includes the summarised results of these examinations, the term 'other species' is taken to include commonly occurring bacteria such as Pasteurella species in the tonsils, or B. subtilis in the nares, none of which is now thought to be of importance in otitis.

TABLE 57

The flora of the ears, nose, tonsils and rectum of dogs suffering from chronic external otitis

	External ears 46		Middle ears 34		Nose 23		Tonsils 23		Rectum 22	
	No.	%.	No.	%.	No.	%.	No.	%.	No.	%.
Pseudomonas	22	(48)	4	(12)	5	(22)	6	(26)	9	(41)
Proteus	18	(39)	4	(12)	6	(26)	9	(39)	14	(64)
Coliforms	11	(24)	6	(18)	4	(17)	14	(61)	22	(100)
Haemolytic streptococci	13	(28)	6	(18)	6	(26)	12	(52)	14	(64)
Non haemolytic streptococci	24	(52)	9	(27)	4	(17)	13	(57)	20	(91)
Staphylococci	26	(57)	16	(47)	22	(96)	14	(61)	15	(68)
'Other species'	34	(74)	14	(41)	17	(74)	21	(91)	22	(100)

Numerals in brackets show percentage of sites infected

Although the nasal carrier rate of staphylococci was high, the rectum appeared to be the commonest source of the other important species, whence they may be readily transferred to the non-infected external ear lesion by the dog scratching. In the case of four dogs suffering from an otitis infected with Pr. mirabilis, this organism was also recovered from lesions in the inguinal region and eyes of three of them.

The figures for the middle ears are misleading as only two dogs showed an inflammatory reaction or exudation in the middle ears at the time of the post-mortem examination. Although neither dog showed clinical symptoms of otitis media, the fact that there was obvious ulceration of the eardrums was probably responsible for the heavy infection with pus formation in the middle ears.

Throughout this thesis the individual ear was taken as one unit, as not all dogs were affected in both ears. In the following attempt to trace the source and spread of infection to the external ears, assuming that it is somewhere in the animal body, each dog was considered to be one unit. The results in Table 58 show the number of dogs, the external ears of which were infected with a particular organism and the number of times the middle ears, the nares, the tonsils and the rectum of each of these dogs was infected with the same strain.

TABLE 58

The commoner bacterial pathogens and their distribution in different tissues

External ear infection	Dogs so affected (Otitis externa)		Percentage of other sites infected with the same organism			
			Middle ears	Nose	Tonsils	Rectum
	No.	%	%	%	%	%
Pseudomonas	13	57	38	39	46	69
Proteus	12	52	25	50	75	92
Coliforms	9	39	24	22	67	100
Haemolytic streptococci	9	39	33	44	56	88
Coagulase positive staphylococci	18	78	33	94	61	67

If the Proteus group is selected to illustrate the significance of these findings, it will be seen that of all the dogs with Proteus infected outer ears, the same organism was present in the middle ears of one out of every four dogs and in the anterior nares, the tonsils and the rectum of every second dog, three out of every four and approximately nine out of every ten dogs respectively. It will be recalled that 47 per cent of the rectal swabs of healthy dogs contained Proteus species and that Pr. mirabilis was not present in the healthy middle ears, nose and tonsils.

The Pr. mirabilis strains from infected tissues were classified by means of the Dienes' phenomenon into a number of antigenic types to determine whether or not the strains from the ears, nares, tonsils and recta of otitic dogs were similar. Fifteen dogs were chosen of which 10 were suffering from bilateral otitis and 5 from unilateral otitis, all of which were infected with Pr. mirabilis. The results showed that all the strains were of the same antigenic type whether or not they were from the outer or middle ears, or both, of the same dog. It will be convenient, therefore, to use the term 'Ear', in Table 59, to indicate the antigenic type that was common to the middle and outer ears of the same dog.

TABLE 59

The spread of Proteus in cases of chronic otitis

Sites infected with Pr. mirabilis				Number of dogs so infected
Ear	Nose	Tonsil	Rectum	
+	+	+	+	4
+	+	+	(+)	2
+	-	+	+	3
+	-	-	+	4
+	-	-	-	2
15	6	9	13	15

NOTE: The symbol + indicates antigenically similar strains
The symbol (+) indicates antigenically dissimilar strains

The results in Table 59 show that in 6 of the 15 dogs suffering from chronic otitis, with an associated Pr. mirabilis infection, the same species was isolated from the ears, nose, tonsils and rectum, four of the six rectal strains being antigenically identical. In addition, antigenically similar strains to the ear strains were isolated from the nares of 6, the tonsils of 9 and the rectum of 11 of the 15 affected dogs.

The method by which the 67 otitic strains of Pr. mirabilis

Pr. mirabilis in Part II were subdivided into 14 antigenically distinct groups, labelled Groups I to XIV, is described in detail in Part VI.b. In this way the mirabilis strains from different sites of the 15 chronically infected dogs were identified as follows:-

TABLE 60

The types of Pr. mirabilis in the ears, nose, tonsils and recta of chronically infected dogs

	No. of dogs infected in the sites indicated			
TYPE	Ears	Nose	Tonsils	Rectum
Group I	7	3	5	5
III	3	-	1	3
XI	1	-	-	1
XII	1	1	1	1
Untyped	3	2	2	3
Total No. of dogs infected	15	6	9	13

NOTE: The untyped group contains the strains that could not be included in any one of the 14 previously identified groups.

Not only were the mirabilis strains from 12 of the 15 dogs in 4 of the 14 previously identified groups, but most were in Group I, as were 42 per cent of the 67 typable strains from the infected ears in Part II of this thesis.

The results in Tables 59 and 60 show that in 15 cases of chronic otitis where the outer and middle ears, or the outer ear only were infected with Pr. mirabilis, an antigenically similar strain was present in the nose and tonsils of 6 and 9 dogs respectively. It will be noted that Pr. mirabilis was isolated from the rectal swabs of 13 of the 15 dogs and that 11 of the strains were antigenically identical.

Otitis media in the dog is extremely rare (Burgess, 1956) and the fact that Pr. mirabilis was recovered from the tonsils of only 9 of the 15 cases of chronic external otitis suggests that the condition is not due to an ascending infection from the naso-pharyngeal region. In these dogs, the presence of Proteus in the anterior nares and tonsils is probably secondary to an ear infection and is the result of contamination. It will also be recalled that Pr. mirabilis was present in the intestines of 37 per cent of the clinically healthy dogs but not in the nares and tonsils.

It is suggested, therefore, that otitis in dogs is due to a lesion, of unknown origin, in the epithelium of the external acoustic meatus which becomes infected, in time, with a variety of organisms. With Pr. mirabilis and also perhaps with a number of other bacteria, the intestine is probably the main reservoir of infection. That other external sources may be responsible, e.g. water, soil and sewage, is shown by the fact that Proteus species were recovered from the external ears of 2 of the 15 dogs, although they were not present in the anterior nares, tonsils or rectum of either animal.

PART III - SUMMARY:

The flora of the ears, nares, tonsils and recta of a number of chronically affected dogs has been discussed and attention has been drawn to the part played by Ps. aeruginosa and Pr. mirabilis in the more resistant types of otitis. It was emphasised that a very high proportion of these dogs became infected with one or both of these organisms either during, or just after, a course of treatment, and that the other bacterial species were, by comparison, of secondary importance.

In an attempt to trace the source and spread of infection, 15 dogs were chosen, all of which were infected in one, or both, ears with Pr. mirabilis. Although Pr. mirabilis was not always present in the nose and tonsils of such cases, the strains were when present antigenically similar to those of the outer ears. On the other hand, most of the rectal swabs were positive and all but two of the strains were similar to the ear strains. This would suggest that most Pr. mirabilis strains are of faecal origin, as may be Ps. aeruginosa and a number of other bacteria.

PART IV

The aetiology of otitis

Various factors are said to predispose to otitis in man and in animals.

It is often said that the initial lesion is caused by irritation of the epithelium of the external acoustic meatus by foreign bodies such as grass awns, dirt, wax and ectoparasites. Other important factors are skin conditions, tumours, generalised infections, allergy, vitamin deficiencies and a low plane of nutrition. Some workers consider that the invading organisms are chiefly responsible for the onset of otitis of which Ps. aeruginosa, Pr. mirabilis, E. coli, Staph. aureus, haemolytic streptococci and certain fungi are probably of most importance.

Collins (1943) summarises the position by stating that "anything which favours the growth of organisms and maceration of the skin will give rise to the condition".

During the four-year period of this survey which involved the examination of material from upwards of 500 dogs, it soon became evident that some of these factors were present in a large number of affected animals. These were classified as follows:-

Skin lesions: Animals clinically affected with dermatitis, pruritus, eczema or conjunctivitis. Also included were cases of labial dermatitis, pedal eczema and anal adenitis.

Ectoparasitic infestation:

Dogs clinically infested with lice, fleas or Otodectes species. A few cases of parasitic mange were also included, although it had not always proved possible to identify the causal parasite.

Foreign bodies:

These included grass-awns, warts, tumours and trauma.

Debilitating conditions:

Generalised infections. Distemper and hard-pad were classified with other conditions including nephritis, tonsillitis and fits.

No History: All the dogs in this group were unaffected by the predisposing factors in the four previous groups and, apart from otitis, appeared to be clinically healthy.

The investigation concerned two groups of dogs. Group I consisted of 351 animals suffering from otitis which were examined before treatment only. Group II included the 100 chronically infected dogs, the ears of which were swabbed before, during and occasionally after treatment.

The incidence of predisposing factors in dogs with external otitis is shown in Table 61.

TABLE 61

Exciting and predisposing factors of canine otitis

A. The animals examined

	Group I (Pre-treatment only)	Group II (Chronic otitis)
Number of dogs examined	351	100
Number of dogs with a history of predisposing factors	196 (56)	67
Number of dogs with no history	155 (44)	33

This shows that 67 per cent of the dogs with chronic otitis also suffered from one, or other, of the predisposing factors referred to above. The fact that more than half of the dogs in the unselected, pre-treatment group were similarly affected suggests that there may be some connection between the onset of otitis and the presence of one, or more, of the predisposing factors.

TABLE 62

Exciting and predisposing factors of canine otitis

B. Skin lesions, ectoparasitic infestation and other factors

Group and number of dogs examined		Skin lesions	Ectoparasites	Other factors
Group I	351	149 (38)	45 (13)	15 (14)
Group II	100	59	14	10

That 38 per cent of the unselected cases and 59 per cent of the chronically infected dogs were suffering from skin lesions suggests that this is the most important predisposing factor to external otitis.

Although ectoparasitic infestation appears to be less significant, it cannot be dismissed as a contributory factor to otitis as 13 per cent and 14 per cent respectively of the dogs in Groups I and II were so affected; nor is it more likely to give rise to the chronic types of otitis.

The other predisposing factors, on the other hand, together accounted for only 15 of the 351 unselected animals and 10 of the chronically infected dogs.

The number of dogs clinically affected with the various conditions is shown in Tables 63, 64 and 65.

TABLE 63

Dogs affected with skin conditions

Number affected in the unselected group 149 (38)
Number affected in the chronic group 59 (59)

Condition	Number of dogs affected: Unselected group	Chronic group
Eczema	37 (11)	15
Dermatitis	54 (15)	18
Pruritus	32 (9)	6
Labial dermatitis	21 (6)	10
Pedal eczema and interdigital cysts	29 (8)	10
Anal adenitis	23 (7)	12
Conjunctivitis	37 (11)	15
Dogs examined	351	100

The percentage figures, in brackets, are based on the total number of dogs examined

The results in Table 63 show that no single condition predominated in the group of skin diseases from which a significant number of dogs with otitis were suffering.

TABLE 64

Dogs affected with ectoparasites

Number affected in the unselected group	45	(13)
Number affected in the chronic group	14	(14)

Condition	Unselected group		Chronic group
Fleas	8	(2)	5
Lice	10	(3)	2
Otodectes species	21	(6)	6
Parasitic mange	7	(2)	2
Dogs examined	351		100

The percentage figures, in brackets, are based on the total number of dogs examined

Many authors have stressed the part played by Otodectes cyanotis var canis in cases of canine otitis. Kauffman and Frost (1949) reported that of 50 dogs suffering from otitis, 56 per cent showed the presence of Otodectes species. Jennings (1953) is of the opinion that ear mites are generally few in numbers, in canine otitis, and that in most cases the parasites are acquired from cats. While Holmes (1933) and Pugh (1947) consider that Otodectes species play an important part in otitis, Georgi (1952) has shown that they are rarely present in such cases.

The incidence of ear mites in cats was described by Beresford-Jones (1955) as being between 20 and 28 per cent, whereas in dogs the figure was 2.5 to 3.5 per cent.

It is, therefore, interesting to find that in this work ear-mite infestation was the commonest parasitic condition, although the figure of approximately 6 per cent is little different from that of Beresford-Jones (1955) for normal dogs.

TABLE 65

Dogs affected with other predisposing factors

Number affected in the unselected group	50 (14)
Number affected in the chronic group	10 (10)

Condition	Number of dogs affected: Unselected group	Chronic group
Distemper or hard pad	16 (5)	-
Nephritis	15 (4)	5
Helminthiasis	13 (4)	3
'Fits'	5 (1)	-
Grass-awns	1 (0.3)	1
Trauma and tumours	7 (2)	3
Dogs examined	351	100

The percentage figures in brackets are based on the total number of dogs examined

None of these factors (Table 65) would appear to be of aetiological significance. Although grass awns were found in only one of the 351 dogs in the group of unselected cases, it is nevertheless possible for foreign bodies of this nature to give rise to an inflammatory reaction in the external ear. The extent of the tissue damage will not always be sufficient to give rise to a 'bacterial otitis', as the offending agents are readily dislodged and the lesion quickly heals.

The correlation between the commoner micro-organisms in infected external ears and the more important predisposing factors to otitis

Investigations were made to determine the possible connection between the organisms in affected ears and the presence of skin lesions and other predisposing factors, as the incidence of Ps. aeruginosa and Pr. mirabilis was shown to increase as the otitis became more chronic. The summarised findings are compared with those of the external ears of healthy dogs in Table 66.

Three groups were chosen, the first of which consisted of 35 healthy dogs. The second group included 351 animals that were swabbed before treatment only, and in the third group were the 100 dogs with chronic otitis.

TABLE 66

Correlation of the bacterial findings and the more important predisposing factors

	Percentage of dogs in each category infected						
		Unselected group 351 dogs			Chronic group 100 dogs		
Organism	Healthy dogs %	History free %	Ecto-parasites %	Skin lesions %	History free %	Ecto-parasites %	Skin lesions %
Pseudomonas	-	10	11	19	13	14	34
Proteus	-	14	18	22	13	21	41
Coliforms	6	7	22	15	10	21	29
Beta haemolytic streptococci	3	14	22	24	7	21	46
Other streptococci	30	26	22	21	29	14	24
Staphylococci	54	70	58	71	81	86	64
Diphtheroids	16	11	11	11	19	21	15
Pityrosporum	36	22	29	22	61	57	36
Total number of dogs in each category	35	155	45	149	31	14	59

The results in Table 66 show that Pseudomonas and Proteus species, which were never present in the ears of normal dogs, were equally prevalent in the infected ears of the other two groups provided that the dogs were not clinically affected with a skin condition. When skin lesions were present elsewhere on the body, the incidence of both Pseudomonas and Proteus rose sharply, particularly in the chronic group. The incidence of coliform organisms was similar to the above but haemolytic streptococci were much commoner in the chronic group when skin lesions were also present. In general, there was little difference between the two groups as regards the incidence of the other bacterial species.

It is also interesting to observe that staphylococci and Pityrosporum species are not influenced by the presence of either skin conditions or ectoparasitic infestation.

These results are illustrated, in graph form, in Diagrams 1, 2 and 3. They are included to emphasise the fact that the important Gram-negative bacteria are much commoner in dogs with skin lesions than staphylococci and Pityrosporum species which are more frequent in dogs that are free of skin complaints. It must also be noted that the overall picture shows that the relative frequencies of the different bacterial species remains unchanged, irrespective of the histories of the animals concerned.

In general terms this means that, while the reservoirs of infection remain unchanged, the extension of a skin complaint to

to the epithelial lining of the auditory meatus provides the Pseudomonas-Proteus group of organisms with the ideal environment for their rapid multiplication.

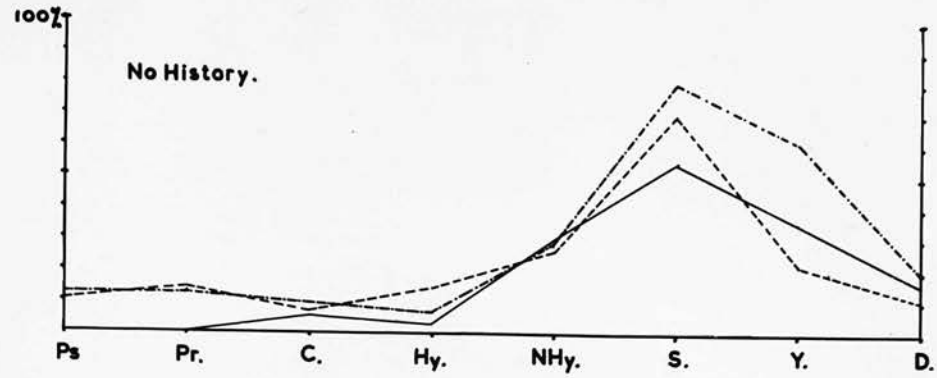
PLATE 3.

Diagrams 1, 2 and 3.

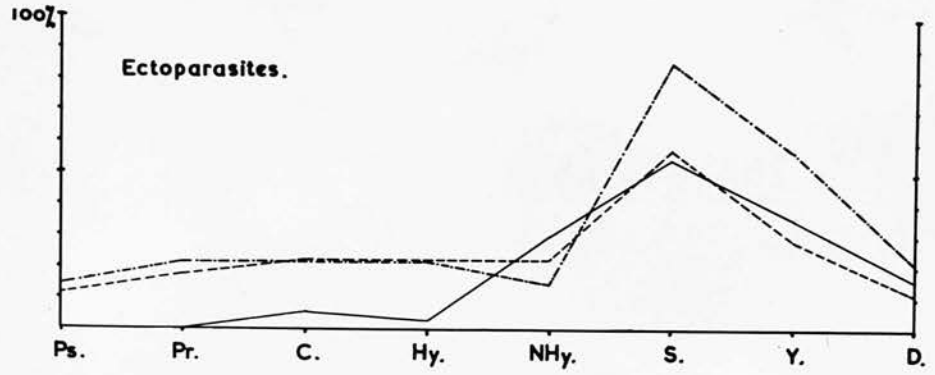
The significance of ectoparasitic infestation and skin diseases in dogs suffering from external otitis.

Note: The terms "Ectoparasites" and "Skin lesions" are used to describe those dogs which were clinically affected with either of these conditions at the time when they were also suffering from otitis.

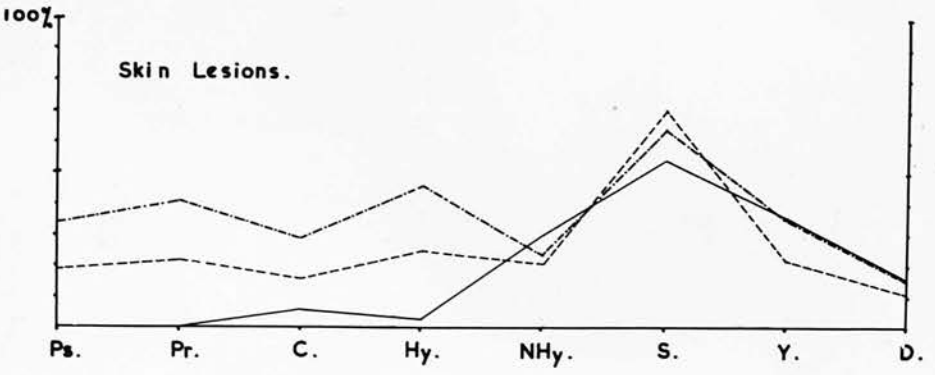
"No history" refers to dogs which were not suffering from a condition that might predispose to otitis.



GRAPH 1.



GRAPH 2.



GRAPH 3.

Legend :-

Ps. = Pseudomonas.
Pr. = Proteus.
C. = Coliforms.
Hy = Haemolytic strept.
NHy. = Non-haemolytic strept.
S. = Staphylococci.
Y. = Pityrosporum.
D. = Diptheroids.

——— OTITIS - Chronic Group. 100 dogs.

- - - - - - - - OTITIS - Unselected Group. 351 dogs.

——— HEALTHY - Normal Group. 35dogs.

PART IV - SUMMARY:

Individual susceptibilities:

There was no reason to suspect that the age or sex of the animal was, in any way, connected with the onset of the condition, even although dogs outnumbered bitches by approximately two to one. That most of the dogs were Spaniels is probably due to the conformation of the ear which is both overlapping and extremely hairy.

Foreign bodies:

In Spaniels, as in other dogs with hairy, overlapping ears, the dark, unventilated external meatus is an ideal receptacle for debris such as desquamated epithelial cells, wax and other secretions. Dirt, hair and foreign matter including grass awns may also be trapped in such circumstances causing irritation and subsequent infection by contaminant bacteria. Grass awns were thought to be of little importance, as ear complaints of this nature are readily alleviated and seldom require to be treated. Trauma, warts and other growths were probably equally unimportant, although a number of cases of benign and malignant tumours were noticed.

Nutritional factors:

Undernourishment, vitamin deficiencies and other nutritional disorders were not considered, although it is realised that they may lead to certain skin conditions which may predispose to otitis.

Debilitating conditions:

A number of local and general infections were considered but it is doubtful if they influence the onset or the course of otitis.

Ectoparasitic infestation:

Although there was no suggestion that the ectoparasites acted as vectors or even as mechanical agents in the transmission of infection, there was evidence to show that they were related to the milder forms of otitis. The most probable explanation of the role played by the ectoparasites, especially the ear mites Otodectes cyanotis, is that they irritate the external ear canal and cause the dog to scratch. This gives rise to a break in the skin surface which, in turn, allows the entry of contaminant organisms. Cultures prepared from these cases usually yielded a heavy growth of coagulase positive staphylococci and/or Pityrosporum species.

Skin lesions:

The importance of skin complaints, as predisposing factors to external otitis, was suggested by the fact that 38 per cent of unselected cases and 59 per cent of chronic otitis cases were clinically affected with some form of skin disease. It appeared possible that an extension of such a condition to the external acoustic meatus might give rise to the initial lesion of otitis which may, in turn, become infected either by commensal staphylococci or Pityrosporum species or by contamination with

with Gram-negative rods such as Ps. aeruginosa and Pr. mirabilis.

The incidence of Pseudomonas and Proteus was highest in long-standing cases of otitis, a number of which only became infected during treatment.

The invading organisms:

Compared with skin complaints, the invading organisms appeared to be of secondary importance as predisposing factors to otitis, although there was evidence to suggest that the nature of the lesion was related to the identity of the predominant bacterial species in infected ears.

Staphylococci and Pityrosporum species were commonest in dogs where the only predisposing factor to otitis appeared to be the presence of ear mites, whereas the highest incidence in infected ears of Pseudomonas and Proteus was in dogs with skin complaints.

PART V

Histopathology of the external auditory meatus

As the presence of skin lesions as a contributory factor to otitis has been emphasised in previous chapters, it was decided to investigate the tissue changes in the affected ears of a selected number of cases of external otitis.

Sections which were prepared from both healthy and affected dogs included material from (a) the outer ear at the level of the external meatal opening, (b) the auditory canal half way along its length and (c) that portion of the meatus which lies immediately proximal to the right angle bend of the canal before it reaches the tympanum.

Although it was hoped to be able to correlate the bacterial findings with the type of tissue reaction, the opportunity was also taken to consider, in more general terms, the abnormalities present and their probable relationship to otitis.

The healthy external ear

The pinna and the outer part of the external acoustic meatus

The conchal cartilage in the dog is unusually large and is invested on both sides with skin, the structure of which is typical of that of the rest of the body. The inner surface contains fewer hairs than on the outside although this depends, to a large extent, on the breed of the animal. The most

most superficial skin structure is the epithelium which is much thinner than the underlying corium and consists of five distinct layers of cells namely, the stratum corneum, stratum lucidum, stratum granulosum, stratum spinulosum, and stratum cylindricum, in that order. Deeper to this lies the corium, or dermis, which is described in two parts, the more superficial of which contains a mixture of delicate reticular fibro and fibro-elastic tissue; the rest being an interweaving network of collagenous and elastic fibres. In contrast to this is the hypo-dermis, or subcutis, which consists of a much looser meshwork of elastic fibres, the spaces in which often contain much adipose material.

In the human external ear sweat glands may occasionally be seen in the hairier parts of the pinna but not in the canal itself, whereas both sebaceous glands and ceruminous glands are frequently found at all levels.

In this work there was no evidence of true sweat glands in the external ears of healthy dogs although sebaceous glands were very numerous in both the cartilagenous and osseous parts of the external meatus. In sections of the more distal parts of the outer ear other glandular structures which resembled the ceruminous glands of other animals were seen in the deeper layers of the corium.

The main difference in the tissues of the healthy ear at different levels lay in the distribution of the sebaceous and ceruminous glands. In the outer part, the former were always

always very large and numerous whereas further down the canal they became progressively smaller and fewer in number. This feature is illustrated in Plates, 4, 5 and 6.

Whilst structures resembling ceruminous glands were occasionally seen in the outer parts of the ear and rarely in the more proximal parts, they were always situated deep to the sebaceous glands which invariably lie in the superficial dermal layers.

Changes in the external ears of dogs affected with otitis

The nature of the discharge:

Small amounts of wax from the secretions of the sebaceous glands (Collins, 1951) were usually seen in the lumen of the external meatus of healthy dogs. This was of a golden-yellow colour and a sticky but dryish consistency which is quite different from the moist or oily discharges of infected ears. In cases of otitis in man this oily secretion is thought to be due to increased activity on the part of the ceruminous glands but, in dogs, these glands appear to be too small and too few in number to produce the copious, purulent type of exudate which is so characteristic of Pseudomonas and Proteus forms of otitis. It may be that, in the absence of ceruminous glands, the discharges are due either to the destructive effects of the invading bacteria on the tissue cells or to the intensity of the inflammatory reaction which their presence provokes.

It was shown, however, that a constant feature in the majority of infected ears was the gross hypertrophy of the

PLATE 4.

The healthy external ear.

The auricle or pinna - medial surface.

Subject. One year old black Springer Spaniel dog.

To show: Fine layers of stratum lucidum and stratum corneum. Thin layer, 5 to 6 cells thick, of stratified squamous epithelium and narrow rete mucosum.

Dense connective tissue in the superficial dermal layers, hair follicles and much adipose tissue in the hypodermis.

The open nature of the cutis vera, especially in the region of the subcutis.

Numerous sebaceous glands of considerable size in the superficial dermis with an occasional small modified apocrine gland lying deep to the sebaceous glands.

Enlargement x 125.

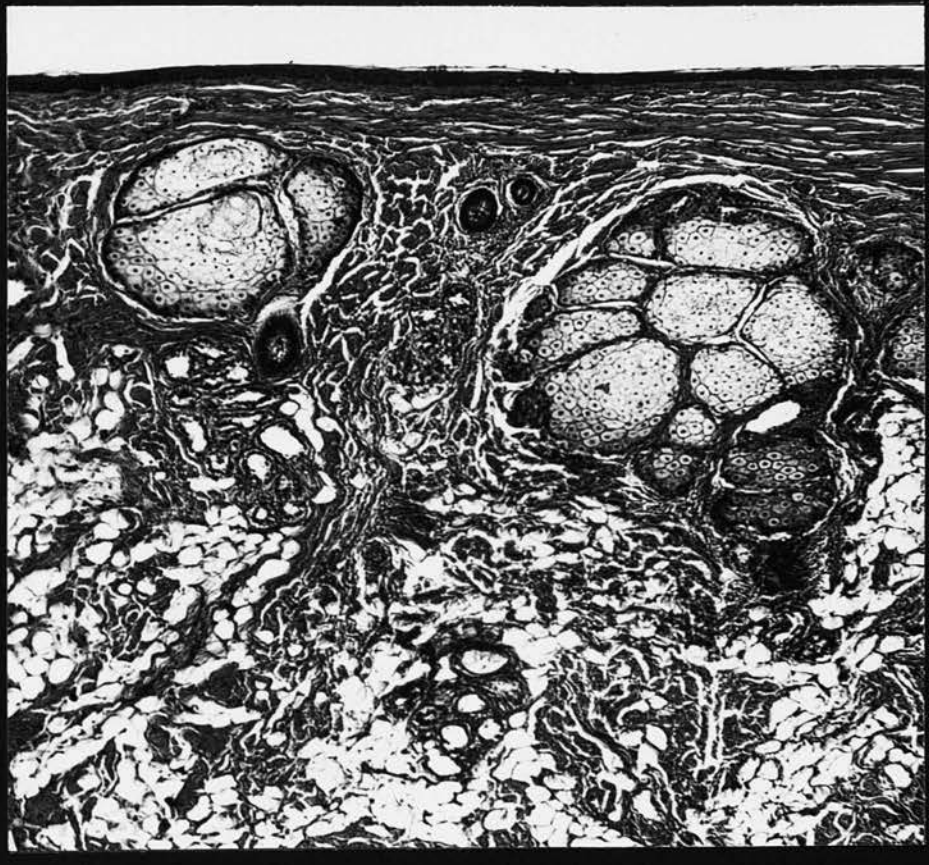


PLATE 5.

The healthy external ear.

The external auditory meatus, immediately proximal to the junction of the conchal and annular cartilages.

Subject. One year old black Springer Spaniel dog.

To show: That the structures are similar to those of the pinna although the sebaceous glands are smaller but equally numerous. They are still lying in the superficial layers of the corium.

The modified apocrine glands are rudimentary or absent.

The connective tissue of the dermis and hypo-dermis is compact and there are fewer deposits of adipose tissue.

The cartilage shows a dense fibrous attachment to the deeper layers of the hypo-dermis.

Hair follicles are still present.

Enlargement x 130.

PLATE 6.

The healthy external ear.

The external auditory meatus, mid way between the 'angle' and the tympanic membrane.

Subject. One year old black Springer Spaniel dog.

To show: That the structures are still very similar to those of the more distal parts of the external ear.

The sebaceous glands are numerous but the follicles are smaller and there is little evidence of the modified apocrine glands.

The connective tissue of the dermal layers is dense and almost completely devoid of fat spaces.

Dense fibrous tissue invests the annular cartilage.

Hair follicles are still to be seen in the deeper parts of the external ear canal.

Enlargement x 120.

the ceruminous glands to form enormous sac-like structures which appeared to be filled with colloidal material. As there was usually a corresponding decrease in the activity of the sebaceous glands, it is perhaps possible that these changes are directly responsible for the type of exudate in the infected meatus.

The epithelium:

In almost every case of canine otitis which was examined histologically changes had occurred in the epithelial lining membrane of the infected meatus. These were usually in the form of shedding of the cells of the stratum corneum with marked hyperkeratinisation of the stratified squamous epithelium and of the hair follicles (Plate 7). Also frequently present were aggregations of inflammatory cells, polymorphs and leucocytes in the prickle cell layer. Occasionally, in the more chronic forms of otitis, hyperplasia of the rete mucosum gave rise to thickening of the epithelium and to the formation of distinct 'rete-pegs' which extended between the dermal papillae into the substance of the corium (Plate 8).

In addition to the thickening of the epithelium and the shedding of the surface cells, ulceration of the lining membrane of the auditory canal was a common feature of the chronically affected ear, particularly those infected with the more important Gram negative organisms (Plate 9). These chronic, ulcerative types of otitis were further characterised by the infiltration of inflammatory cells especially in the superficial dermal layers

PLATE 7.

Tissue reactions in otitis externa.

The epithelium.

Subject. Five year old Spaniel bitch.

Tissue. 'Angle' of the external ear.

History. Sub-acute otitis of six months' duration, associated with Pr. mirabilis.

To show: Hyperkeratinization of the stratified squamous epithelium and hair follicles.

Hyperplasia of the rete mucosum with proliferation of the stratum germinativum, giving rise to 'rete peg' formation.

Inflammatory cell reaction, with polymorphs and macrophages, in the prickle cell layer.

Moderate fibroplasia.

Enlargement x 90.

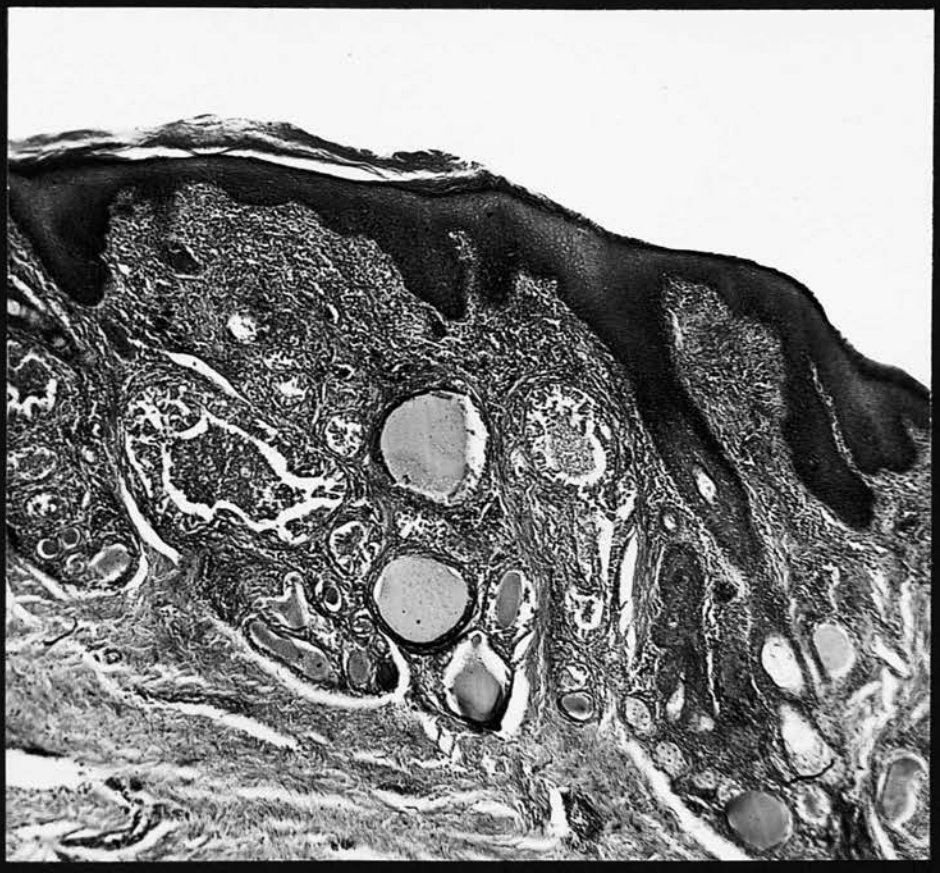


PLATE 8.

Tissue reactions in otitis externa.

The epithelium - formation of 'rete-pegs'.

Subject. Six year old Cocker Spaniel dog.

Tissue. Right 'angle' of the external ear.

History. Chronic otitis of six months' duration with an associated Pr. mirabilis infection. Tympanum perforated.

To show: Exaggerated 'rete-peg' formation due to the proliferation of the cells of the stratum germinativum. This, and ulceration of the epithelium, are fairly common features of Proteus infected ears.

Hyperkeratinization and proliferation of the stratified squamous epithelium giving rise to a bloated appearance of many of the cells.

Extensive polymorph infiltration of the dermal papillae.

Enlargement x 90.

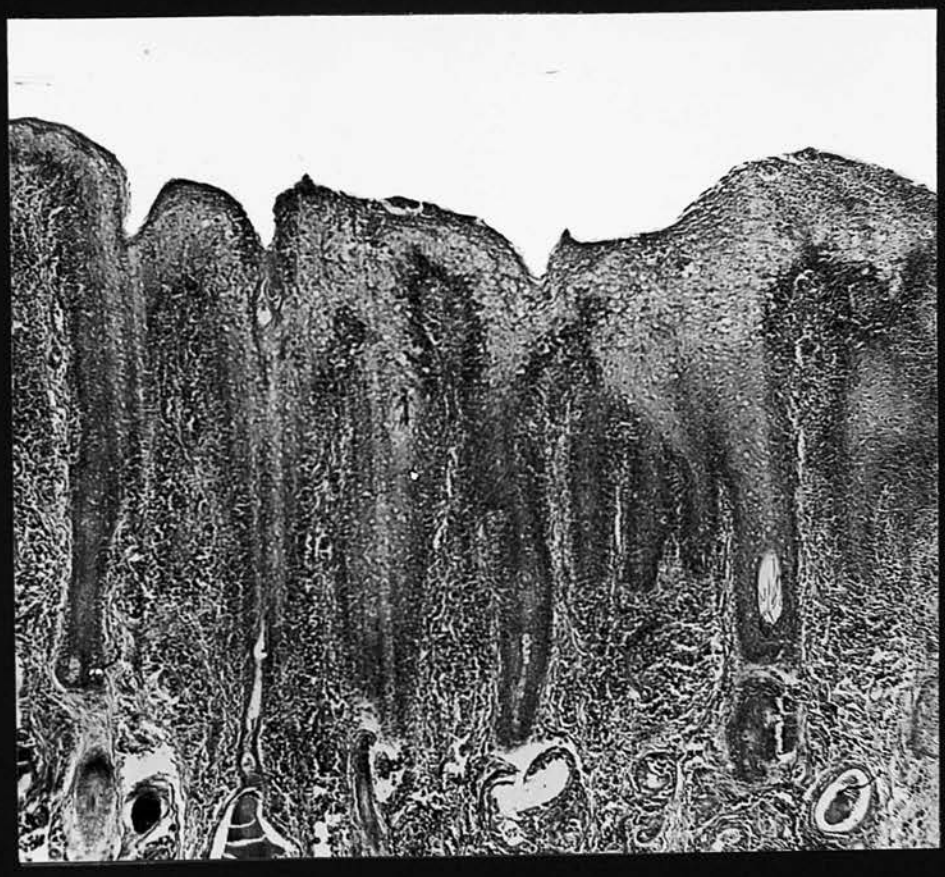


PLATE 9.

Tissue reactions in otitis externa.

The epithelium - ulceration.

Subject. Five year old Springer Spaniel dog.

Tissue. Left 'angle' of external ear.

History. Chronic otitis of four months' standing with an associated Ps. aeruginosa infection. Tympanum intact.

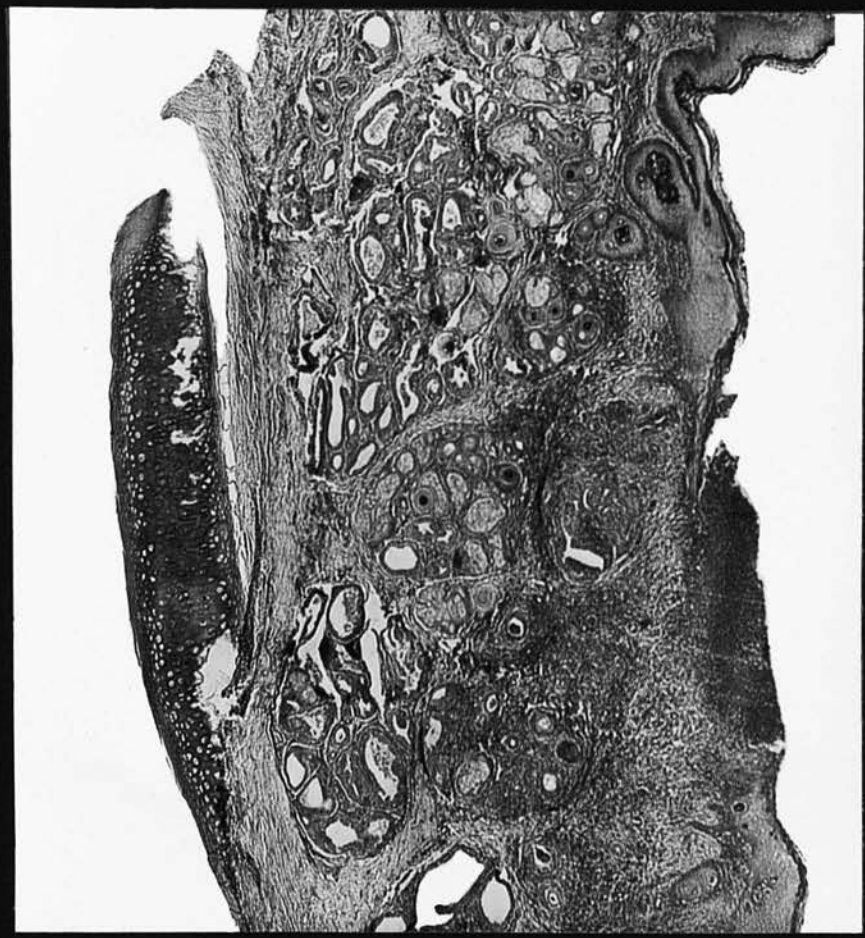
To show: Hyperkeratinization of the stratified squamous epithelium with proliferation of the cells. 'Rete-peg' formation not marked.

Ulceration of the epithelium with infiltration of the superficial dermal layers with inflammatory cells.

Haemorrhagic areas in the vicinity of the ulcer, extending into the corium in the region of polymorph and macrophage infiltration.

Marked glandular activity and a degree of fibroplasia.

Enlargement x 40.



layers immediately beneath the stratum germinativum. Areas of haemorrhage, which were usually to be seen in the ulcerated epithelium, extended into the dermis in the areas of polymorph and macrophage infiltration. Of practical importance was the proliferation of fibrous tissue in the corium which leads to thickening of the subcutaneous tissues and occlusion of the lumen of the external acoustic meatus.

Fibrous tissue:

Robb-Smith (1954) considers that the normal connective tissue consists of an interlocking network of collagen, reticulum and elastic fibres lying in a non-fibrillary matrix of ground substance throughout which are scattered a variety of isolated cells. These include elongated fibroblasts, lymphocytes, plasma cells, neutrophils, histiocytes, basophils, tissue mast cells and, in the subcutis, a number of fat cells.

Asboe-Hansen (1954) is of the opinion that fibroblasts predominate numerically among the cells of the dermal connective tissue in an adult body. The role of the fibroblasts is explained by the fact that whenever a tissue is exposed to injury it responds with an inflammatory reaction, the course of which depends on (a) the nature of the irritant, (b) the tissue affected and (c) the duration of the irritation (Zachariae, 1954).

The most significant feature of the more chronic inflammatory reactions is the proliferation of fibroblasts and the gradual increasing production of fibrous tissue (Plate 10). Dense

PLATE 10.

Tissue reactions in otitis externa.

The connective tissue - fibroplasia.

Subject. Three year old Alsatian bitch.

Tissue. Left ear, mid-way between the 'angle' and the external opening of the ear canal.

History. Sub-acute otitis of four weeks' duration with an associated staphylococcal infection.

To show: The hypodermal connective tissue layers.
The proliferation of the elongated fibroblasts and a gradual increase in the formation of fibrous tissue.
Occasional cells in a sub-chronic inflammatory reaction.

Enlargement x 1000.

Dense fibrous strands are seen to extend from the deeper dermal layers throughout the corium and appear to be replacing the areas normally occupied by adipose tissue and connective tissue matrix. In a number of such cases the resulting increase in thickness gives rise to fibroma-like masses which tend to occlude the lumen of the ear (Plate 11).

The tissue mast cell:

As far as skin is concerned, the presence of mast cells has been demonstrated in man, mouse, rat, cat, dog, ox, pig, guinea pig and rabbit, as well as in other vertebrates.

Although they do not occur in the early embryo, first appearing toward the end of embryonic life, they later occupy locations which, though characteristic for the species, differ markedly from one species to another. Moreover, they often appear first and are later extraordinarily numerous in the ear (Riley, 1956).

The same author (Riley, 1955) has described two types of cell, namely, the tissue mast cell which is found almost exclusively in the connective tissue and the rarer blood mast cell, or mast leucocyte, which takes origin in the marrow and enters the peripheral blood with the other leucocytes.

Although the role of the mast cells is as yet imperfectly understood, they are known to store, if not to synthesise, heparin (Asboe-Hansen, 1954) and histamine (Riley, 1953) and it has been shown that, as far as skin is concerned, the bulk of

PLATE 11.

Tissue reactions in otitis externa.

The dermal connective tissue - fibrous tissue reaction.

Subject. Four year old Cocker Spaniel dog.

Tissue. External meatus of left ear immediately inside the the opening of the auditory canal.

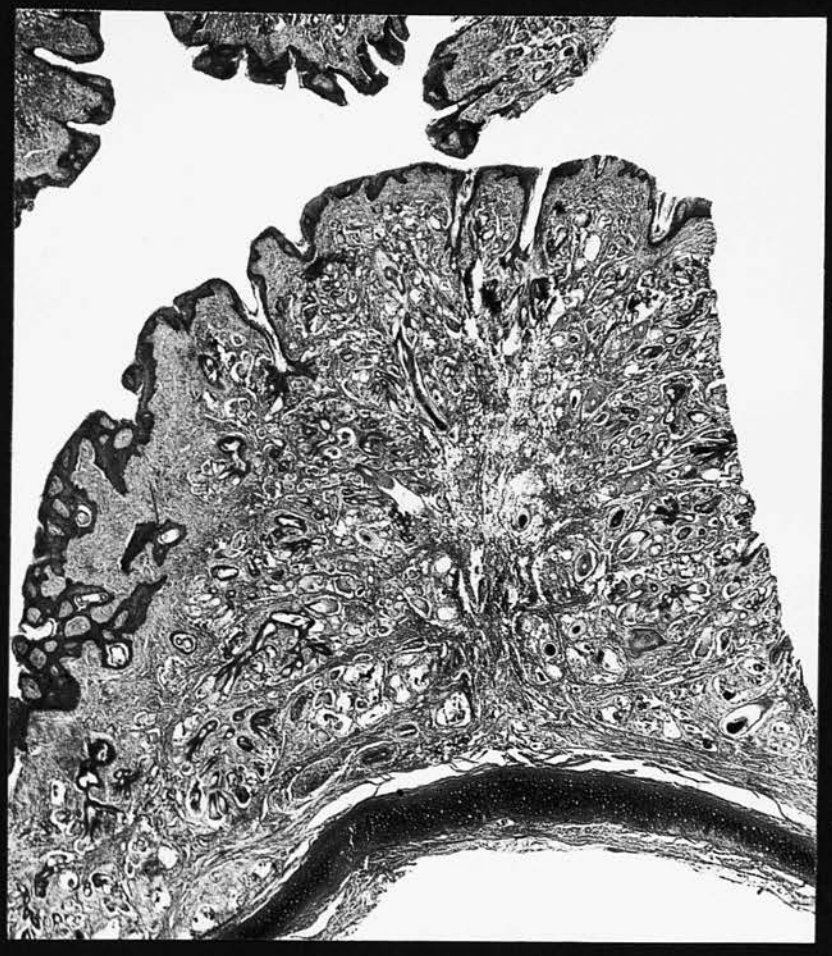
History. Chronic otitis of 4 months standing, associated with a mixed bacterial infection. Ulceration of meatal lining and of the tympanum.

To show: The proliferation of the fibrous tissue extending from the deeper dermal layers, throughout the corium. That as the result of marked fibroplasia the lining membrane is very much thickened forming tumour-like swellings that tend to occlude the lumen of the outer ear canal.

The comparatively small amount of glandular tissue reaction.

Note. The lumen of the external acoustic meatus is situated in the top left hand corner of the plate.

Enlargement x 20



of the extractable histamine is situated in that part of the skin which contains most mast cells(Riley, 1956). It is the opinion of Riley and West (1953) that liberated histamine, as the result of injury, may play a part in the mobilisation of the fixed tissue cells which precedes repair. MacDougall (1954) has demonstrated that the addition of mast cells to a tissue culture of fibroblasts favours the production of fibroblasts in the medium.

The theory that mast cells are concerned with maintenance and repair of connective tissue is again advanced by Riley (1954) when he states that if connective tissue is subject to trauma - mechanical, clinical, thermal or bacterial - whereby an acute watery oedema is produced, the mast cells promptly release their metachromatic substance into the tissues. Fibroplasia follows and as recognisable collagen begins to take shape, mast cells once more appear in the reactive zone. Should fibroplasia be unduly protracted as in acute inflammation, the mast cell population increases further.

Larsson (1957) has found that dogs in general and Boxers in particular are predisposed to a variety of tumours including mastocytoma. Other workers have also reported the high mast cell content of canine tissues.

In man the presence of mast cells in many skin diseases has been well described and it is interesting to recall that, in many of these which show dense infiltrations of mast cells, the

the sebaceous glands are reduced in number or are absent altogether.

During the examinations of sections stained by Haematoxylin and Eosin from the ears of dogs affected with otitis, large inflammatory areas were seen in the region of maximum fibrous tissue reaction, particularly in the more chronic cases. As the majority of these 'inflammatory' cells looked like macrophages and in view of what has just been said, it was decided to investigate the true nature of these cells in the ear sections of a limited number of dogs. When it was realised that they were, in fact, mast cells an attempt was made to correlate the tissue reaction, the presence of mast cells and the type and invasiveness of the bacterial pathogens. To do so sections were stained routinely by Haematoxylin and Eosin, by Gram and by Gomori's (1950) Aldehyde Fuchsin Method.

The position may be summarised by stating that, in normal ears and in a number of chronically infected ears, the mast cells were not conspicuous numerically. The typical picture, which is represented in Plate 12, shows a chronically infected ear with only a small number of vacuolated, adult mast cells in the superficial dermal layers. This vacuolation of the cytoplasm with a tendency to disruption, in some cases, suggests the action of histamine liberators on the mast cell and that the lesion is active.(Drennan, 1951). The metachromatic granular nature of the cell suggests its maturity.

PLATE 12.

Tissue reactions in otitis externa.

The response to inflammatory stimuli - the tissue mast cell

Subject. Five year old Springer Spaniel dog.

Tissue. Angle of right ear. Stained by Gomori's aldehyde-fuchsin method.

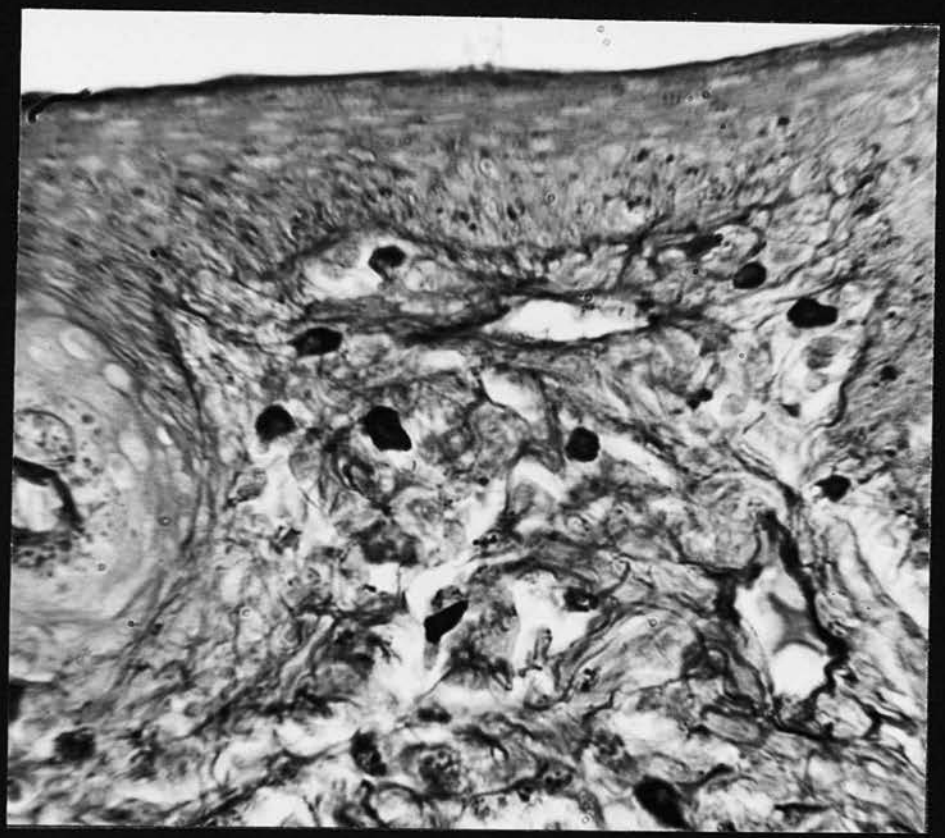
History. Bilateral otitis of four months' standing with an associated Ps. aeruginosa infection.

To show: The tissue mast cells scattered loosely throughout the superficial dermal layers. A similar picture is seen in healthy ears.

The large irregular shape of the majority of cells in this region, most of which contain metachromatic granules. This suggests that the cells are mature.

Note particularly the vacuolated appearance, with a tendency to disruption in some cases, of the cytoplasm. This is probably due to the action of histamine liberators on the mast cell.

Enlargement x 700



In a number of chronic cases large irregular pockets of mast cells were seen in areas of fibrous tissue reaction (Plate 13). As the majority of these cells were vacuolated and as there was also evidence of proliferation of the fibroblasts with a gradual increased production of connective tissue, this suggested that the case in question had been subject to continued irritation. (The case was, in fact, one of four months' standing, the ear being infected with Ps. aeruginosa).

In conclusion it is suggested that the presence of mast cells in the ears of dogs affected with otitis is an indication of a defensive mechanism being brought into play. If the mast cells are associated with the liberation of histamine, this will determine the viscosity of the dermal connective tissue by increasing the rate of diffusion of hyaluronic acid which is a chemically related substance to the heparin of the mast cell.

It may be, therefore, that the activity of the mast cell governs, not only the amount of fibrous tissue produced and the viscosity of the dermal connective tissue, but also the invasiveness and rate of spread of pathogenic micro-organisms.

The glandular structures:

Mention has already been made of a number of skin conditions in man in which a marked increase in the number of mast cells is a constant feature. Some of these, e.g. Poikiloderma vasculare atrophicans also show a reduction in the number of sebaceous glands. As the sebaceous glands are responsible for the

PLATE 13.

Tissue reactions in otitis externa.

The response to inflammatory stimuli - the tissue mast cell.

Subject.
Tissue. As for Plate 12.
History.

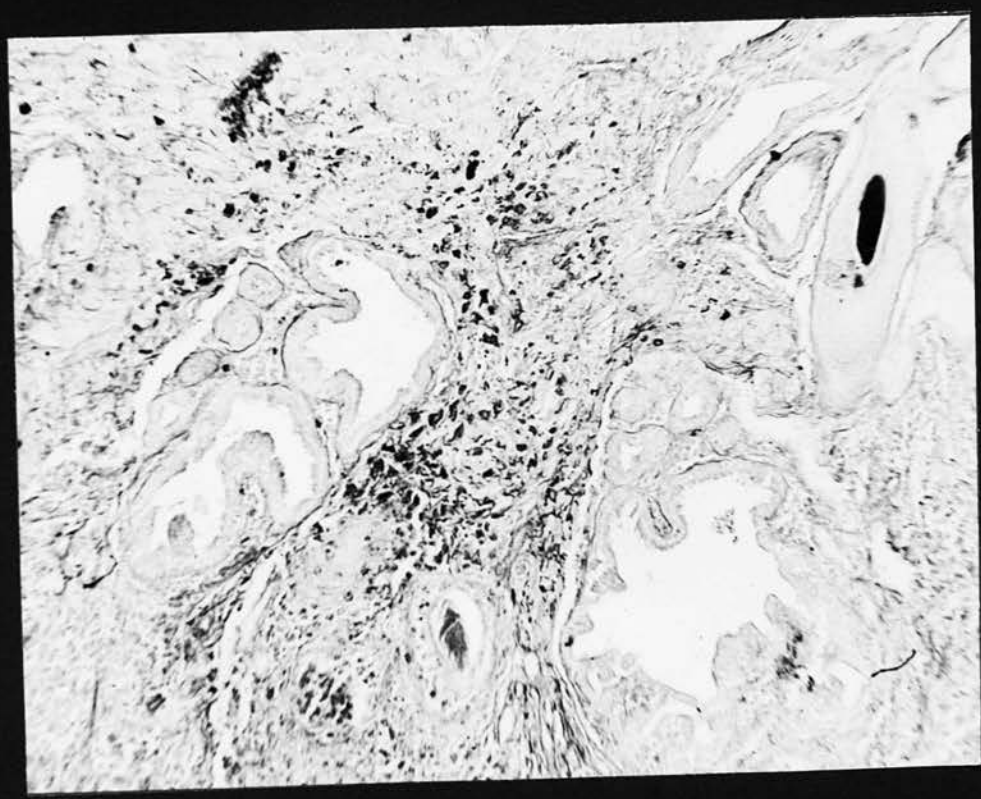
To show: The distribution of the mast cells in the deeper parts of the corium.

Proliferation of fibroblasts and a gradual increased production of fibrous tissue.

Aggregation of tissue mast cells in the dermal connective tissue, particularly in the region of marked fibroplasia.

The presence of the proliferating vacuolated mast cells suggests that the case is one associated with continuous irritation.

Enlargement x 82.



the secretion of the waxy component of the cerumen of the healthy external ear (Collins, 1951) which however is entirely different from the oily or moist discharges of the affected ear, this suggests that in otitis there is a period of inactivity, or regression, on the part of the sebaceous glands (Plate 14).

In the healthy external ear of the dog it has been shown that sebaceous glands are large, fairly numerous structures lying in the superficial layers of the corium, as opposed to the so-called ceruminous type of glands which are seen, only with difficulty, in the deeper dermal layers.

The sebaceous glands (Plate 15) are simple, alveolar, holocrine glands which appear as evaginations of the hair follicle, the membrane of which is continuous with the basement membrane of the gland. The excretory duct of larger glands bears an epithelium resembling the epidermis and is covered by a stratum corneum. According to Trautmann and Fiebiger (1952), the glands in the dog are often club-shaped, coiled and branched with, occasionally, several follicles uniting into a common group with a single opening.

A fairly constant feature of most cases of canine otitis, esprcially the chronic forms, was the fact that the sebaceous glands became smaller and less active, than in normal ears, and that they tended to be displaced in the superficial dermal layers by the numerous dilated ducts of the tubular apocrine glands. (See Plates 7 and 16).

PLATE 14.

Tissue reactions in otitis externa.

The sebaceous glands.

Subject. Twelve year old Golden Cocker Spaniel dog.

History. Chronic bilateral non-suppurative otitis of nine months' duration. Staph. aureus isolated from both ears.

Tissue. Right ear immediately proximal to the external opening of the ear canal.

To show: Sebaceous glands that are still numerous but smaller and less active than in the healthy external ear. (See plates 4, 5 and 6).

Sebaceous glands are superficially placed in the dermis whereas in chronic suppurative types of otitis, especially those associated with Pseudomonas and Proteus infections, they tend to be displaced by the modified apocrine glands to the deeper parts of the corium.

The absence of modified ceruminous glands in this section may account for the fact that the ear discharges were still quite waxy in appearance.

Note. The hyperkeratinization of both the lining membrane and the numerous hair follicles.

Enlargement x 115

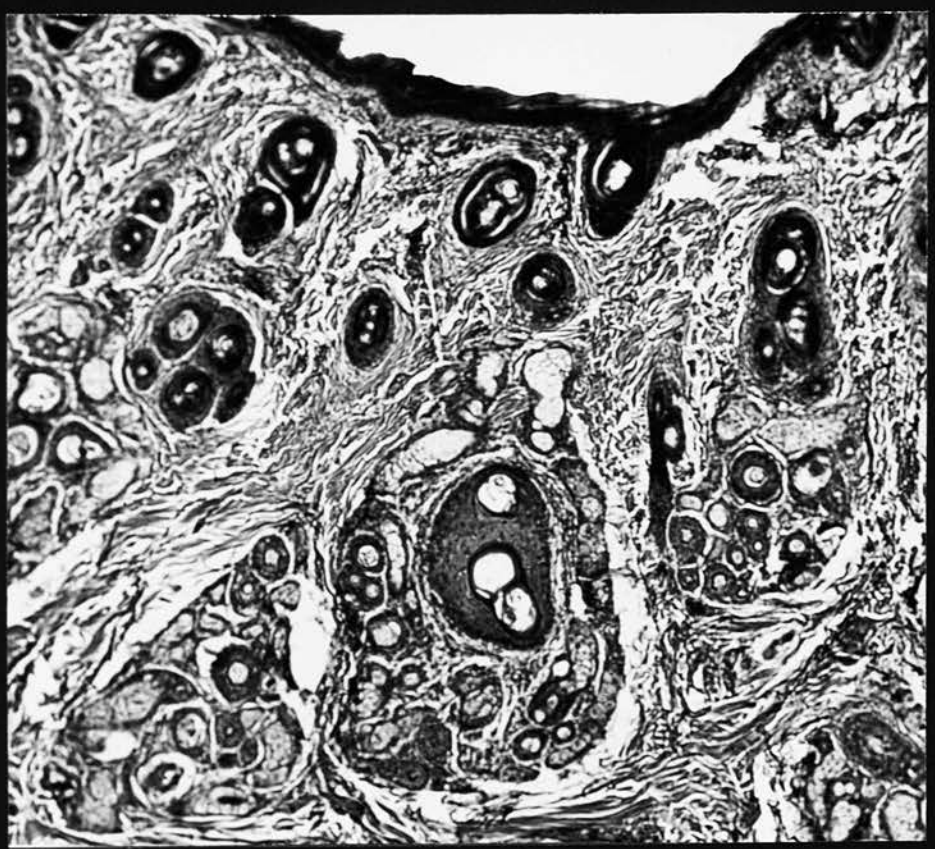


PLATE 15.

Tissue reactions in otitis externa.

The sebaceous glands.

Subject. Five and a half year old Spaniel dog.

History. Sub-acute external otitis of six months' duration.
No obvious bacterial pathogen isolated.

Tissue. Left external ear at level of the 'angle'.

To show: Hyperkeratinization of the cells of the superficial epithelial layers and of the hair follicles, with a tendency to rete-peg formation.
Numerous sebaceous follicles which are large and, for the most part, are still active. They are still situated in the superficial dermal layers although there is already evidence of increased activity on the part of the modified apocrine, or ceruminous, glands.

Note. The association of the sebaceous glands and the hair follicles. Groups of sebaceous follicles usually tend to form a single excretory duct which opens into the hair follicle, deep to the stratified squamous epithelium.

Enlargement x 70

PLATE 16.

Tissue reactions in otitis externa.

The modified apocrine, or ceruminous, glands.

Subject. Thirteen year old Airedale dog.

History. Long standing otitis associated with a mixed bacterial infection including staphylococci, non-haemolytic streptococci and diptheroid bacilli.

Tissue. 'Angle' of right external ear.

To show: Large numbers of so-called ceruminous glands which have increased enormously in size to form cyst-like structures.

These diverticula are frequently distended with an eosinophilic secretion which appears to be of a colloidal or fatty nature.

The cystic ceruminous glands are usually situated in the more superficial dermal layers in long standing cases of otitis, whereas in the more acute forms of otitis they are smaller, less numerous and are to be found in the deeper layers of the corium.

Enlargement x 110

Apocrine tubular glands.

The ceruminous type of glands which are described as being serpentine and rolled up in a ball, and are only occasionally to be seen in the deeper parts of the external canine ear are, in fact, a form of apocrine tubular gland; although their appearance and distribution are quite different in otitis.

In most cases of otitis, and in the chronic forms in particular, the apocrine glands are usually enlarged and actively secreting although in the superficial dermal layers they tend to displace the sebaceous glands by forming numerous cystic tubular dilatations, many of which are distended with an eosinophilic, homogeneous, colloidal substance; while others appear to be quite empty. The differences between these two types of gland are shown in Plate 17, which is of a less advanced case of otitis. This shows the honeycomb appearance of the sebaceous follicle which is invariably to be found in association with a hair follicle. The apocrine type of gland, on the other hand, is more sac-like and is lined by a single row of low cells with flattened nuclei.

Although many of the tubular glands were actively secreting, it is suggested that the presence of large cystic diverticula, in the superficial layers of the corium, is probably due to constriction of the excretory duct, near the opening to the surface of the skin, by the inflammatory reaction which is a feature of most cases of otitis.

Plates 19 to 22, and 23 to 26, are included to show how each sac-like dilatation of the apocrine gland has its own excretory duct which usually opens directly on to the free surface of the skin or/

PLATE 17.

Tissue reactions in otitis externa.

The glandular tissues in affected ears.

Subject. Nine months' old Border Terrier bitch.

History. Long standing unilateral otitis with little evidence of bacterial infection. Pityrosporum species were usually present in small numbers.

Tissue. The epithelium of the annular cartilage of the left external ear.

To show: To the left are small sebaceous follicles with their associated hair follicle. These glands are simple alveolar holocrine glands having a basement membrane that is continuous with the lining of the hair follicle.

To the right is part of a modified apocrine, or ceruminous, gland the lumen of which is enlarged and lined by a row of low cells with flattened nuclei.

Enlargement x 660

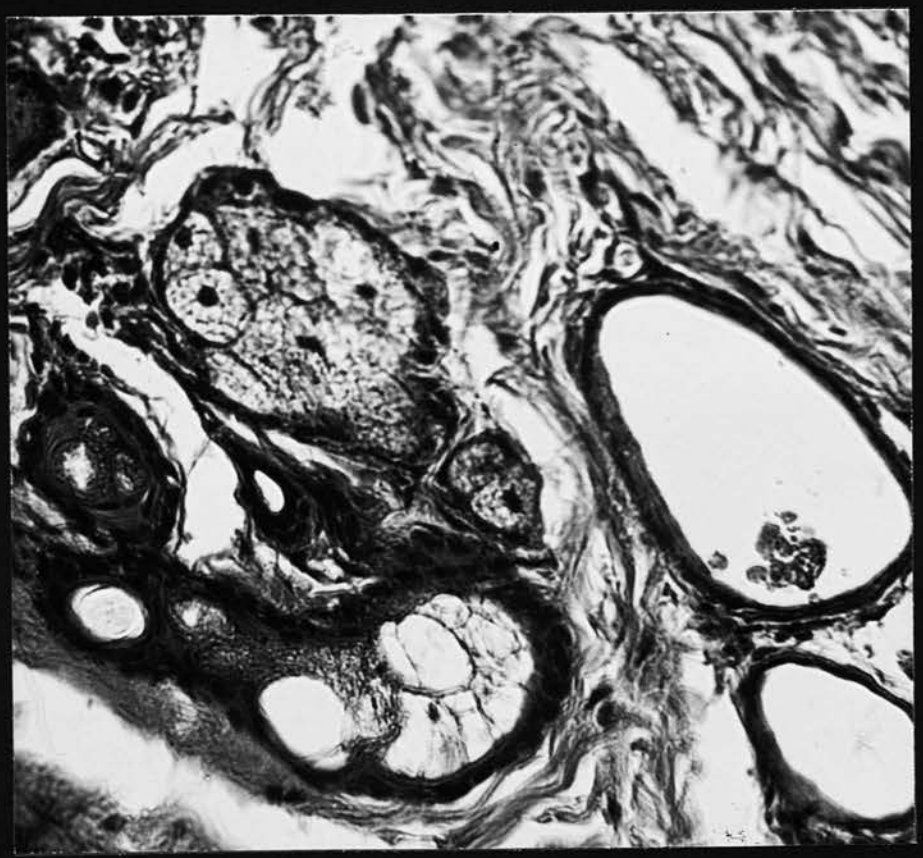


Plate 18.

Tissue reactions in otitis externa.

The ducts of the sebaceous glands.

Subject: 5 year old Springer Spaniel dog.

Tissue: Left angle of the external acoustic meatus.

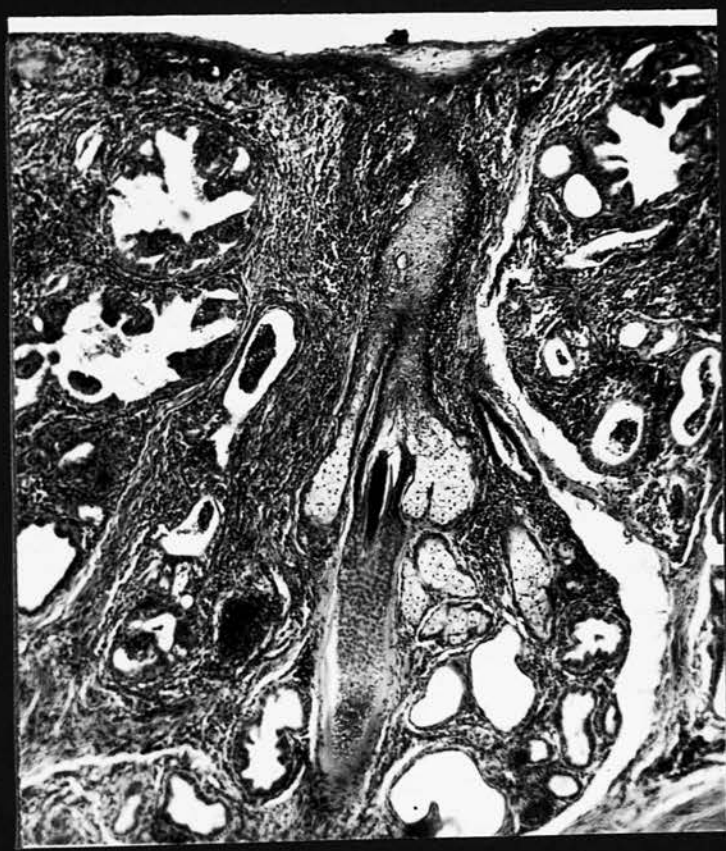
History: Chronic otitis of four months' standing.

To show: That two or more sebaceous glands are frequently attached to each hair follicle.

That the ducts of the sebaceous glands open into the deeper parts of the hair follicle and not, as with the modified apocrine glands, directly to the skin surface.

Note: In a number of ears, the ducts of the sebaceous glands united to form a single opening into the hair follicle.

Magnification x 60.



PLATES 19 and 20.

Tissue reactions in otitis externa.

The excretory duct of the modified apocrine, or ceruminous, glands.

The serial sections of the external ear of a chronic case of otitis show the course of the single excretory duct of the modified apocrine glands. In this instance the duct reaches the surface of the skin close to the opening of a hair follicle.

Enlargement x 90

PLATES 21 and 22.

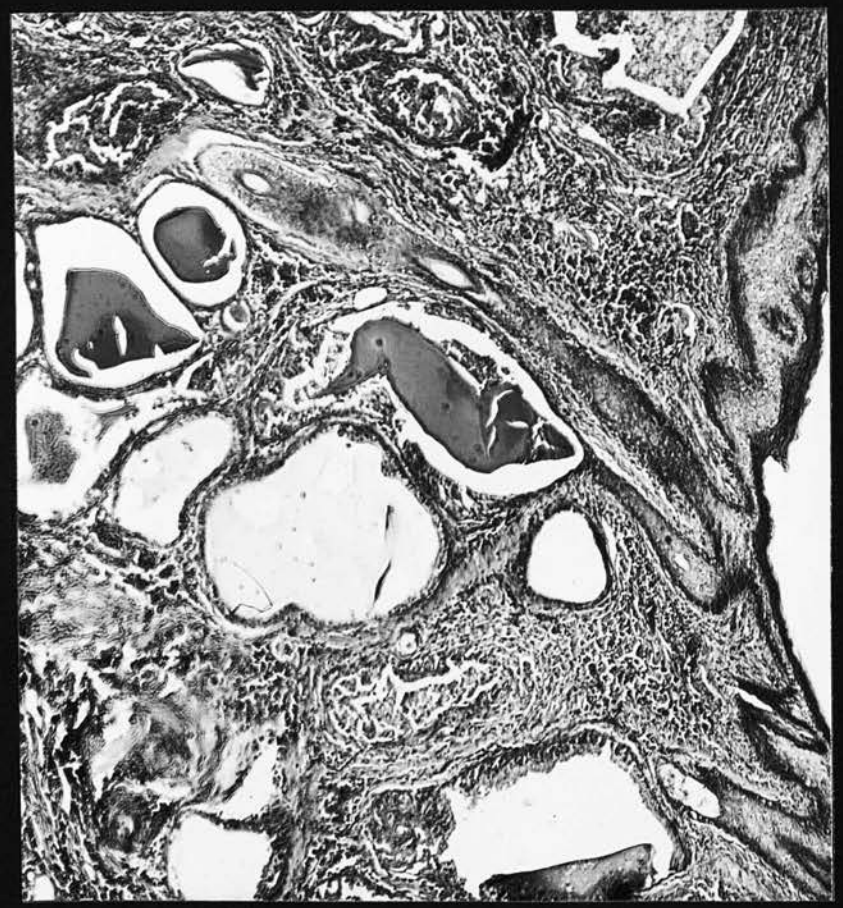
Tissue reactions in otitis externa.

The excretory duct of the modified apocrine, or ceruminors, glands.

These plates show the course of the single excretory duct as it runs parallel to the hair follicle without joining it. Notice the colloidal nature of the secretion in part of one of the coils of the gland.

Each tubular gland appears to have only one duct unlike the sebaceous glands, the follicles of which give rise to small secretory ducts that unite to form a single, large duct. This in turn opens into the deeper parts of the associated hair follicle.

Enlargement x 90



PLATES 23 and 24.

Tissue reactions in otitis externa.

The excretory duct of the modified apocrine, or ceruminous, glands.

The serial sections in the following four plates trace the course of the excretory duct of a modified tubular gland. In this case, however, the duct will be shown to open to the surface at some distance from the nearest hair follicle.

Enlargement x 85

PLATES 25 and 26.

Tissue reactions in otitis externa.

The course of the excretory duct of the modified ceruminous, or apocrine, glands.

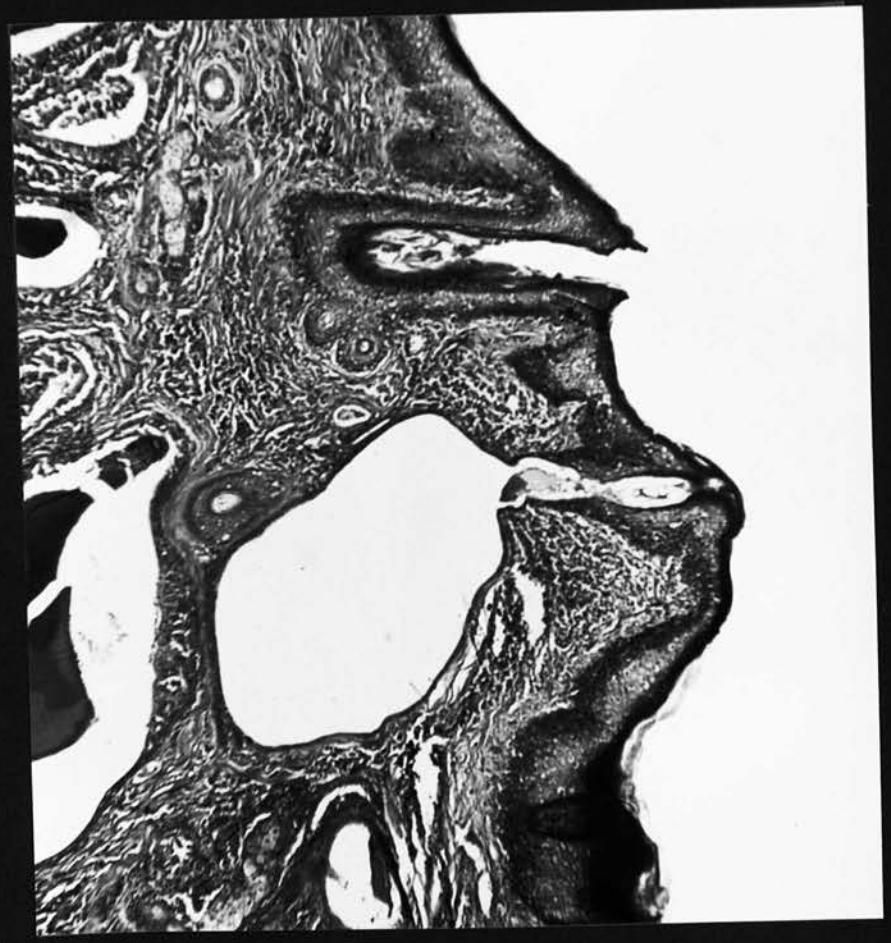
(Continued from Plates 23 and 24).

Enlargement x 85

Notes.

The presence of large numbers of cyst-like diverticula in the superficial dermal layers of otitic ears is probably due to dilatation of the ducts of actively secreting apocrine glands. Further distension may also be due to blockage of the excretory portion of the tube as it reaches the skin surface, by inflammatory changes which are usually a feature of chronically infected ears.

In many of the sections the enormous size of these structures was probably due to the duct, or a loop of the duct, being cut at an oblique angle.



or gains the surface in close proximity to the hair follicles, unlike the sebaceous glands which usually form a single duct into a hair follicle (Plate 18).

There is little doubt that as a result of these examinations, it can be said that the more chronic the type of otitis, especially if infected with either Proteus or Pseudomonas organisms, the more watery is the discharge and the less active are the sebaceous glands.

Tumours:

A number of authors agree that tumours, and in particular tumours of the ceruminous glands, are related to chronic otitis in dogs and cats. (Courthaliac, 1939). Two types of tumour were encountered in this survey, namely those which involved either the ceruminous glands or the sebaceous glands.

(a) Adenocarcinoma of the ceruminous glands:

It will be recalled that the so-called ceruminous glands in the external ear of the dog are specialised sudoriferous glands which are thought to secrete at least one of the components of normal cerumen. The lumena of these tubular structures are large and often contain discs of colloidal material which may impart viscosity to the cerumen.

In tumour formation of these glands (Plate 27) numerous glandular cavities of various shapes and sizes are seen. Within these sac-like structures is much cellular debris, macrophages and polymorphs which according to Ball (1939) indicates that the

the tissue has been the seat of infection and secondary inflammation of external origin. Large plaques of colloidal material may also be present. The remainder of the corium usually consists of dense areas of fibrous tissue with apparently normal ceruminous glands throughout. In the case illustrated in Plate 27 the hyperplastic glandular material is seen to extrude beyond the annular cartilage.

The lining membrane of these grossly distended ceruminous glands gave rise to papillary projections which are very similar to the pattern seen in an intraductal carcinoma of the mammary gland (Plate 28).

Separating the ceruminous glands are strands of adult connective tissue infiltrated by plasma cells, lymphocytes and macrophages. It is also of interest to notice that the associated parotid lymph node showed marked lymphoid hyperplasia without evidence of metastasis (Head, 1955).

(b) Tumours of the sebaceous glands:

In the normal ear sebaceous glands are numerous and mostly of considerable size, the largest of which are situated in the superficial layers of the dermis. Each gland is made up of a variable number of sebaceous follicles which open into an unique excretory canal.

In the tumour of the sebaceous glands (Plate 29) hyperkeratinisation of the stratum corneum was most marked, as was the thickening of the rete mucosum, although there was little evidence

PLATE 27.

Tumours associated with chronic external otitis.

Adenocarcinoma of the modified ceruminous glands.

Subject. Fourteen year old liver and white Springer Spaniel dog.

History. Chronic otitis of six months' standing. Both external and middle ears infected with Pr. mirabilis.

Tissue. Left external ear at the level of the 'angle'.

To show: Gross hyperplasia of the ceruminous glands extending outwards beyond the annular cartilage.

Dense fibrous tissue reaction with a number of apparently normal ceruminous glands in the corium.

Macroscopical examination of the external acoustic meatus showed evidence of thickening of the epithelium in the region of the junction of the annular and conchal cartilages. In the deeper parts of the ear canal the lumen was occluded by a large fibrous proliferating mass.

The right ear, however, appeared to be healthy.

Enlargement x 78

PLATE 28.

Tumours associated with chronic external otitis.

Adenocarcinoma of the ceruminous glands (continued.)

Subject and material, as for plate 27.

To show: The lining membrane of the grossly distended ceruminous glands, giving rise to papillary projections which are very similar to the pattern seen in an intraductal carcinoma of the mammary gland.

Separating the ceruminous glands are strands of adult connective tissue infiltrated by plasma cells, lymphocytes and macrophages.

Note. Examination of the left parotid lymph node showed marked lymphoid hyperplasia without evidence of metastasis.

Enlargement x 45

PLATE 29.

Tumours associated with chronic external otitis.

Tumour of the sebaceous glands.

Subject. Eight year old Border Terrier dog.

Tissue. Left outer ear at the level of the opening of the external acoustic meatus.

History. Otitis of six months' duration associated with a mixed bacterial infection.

To show: Hyperkeratinization of the stratum corneum

Thickening of the rete mucosum but without rete-peg formation.

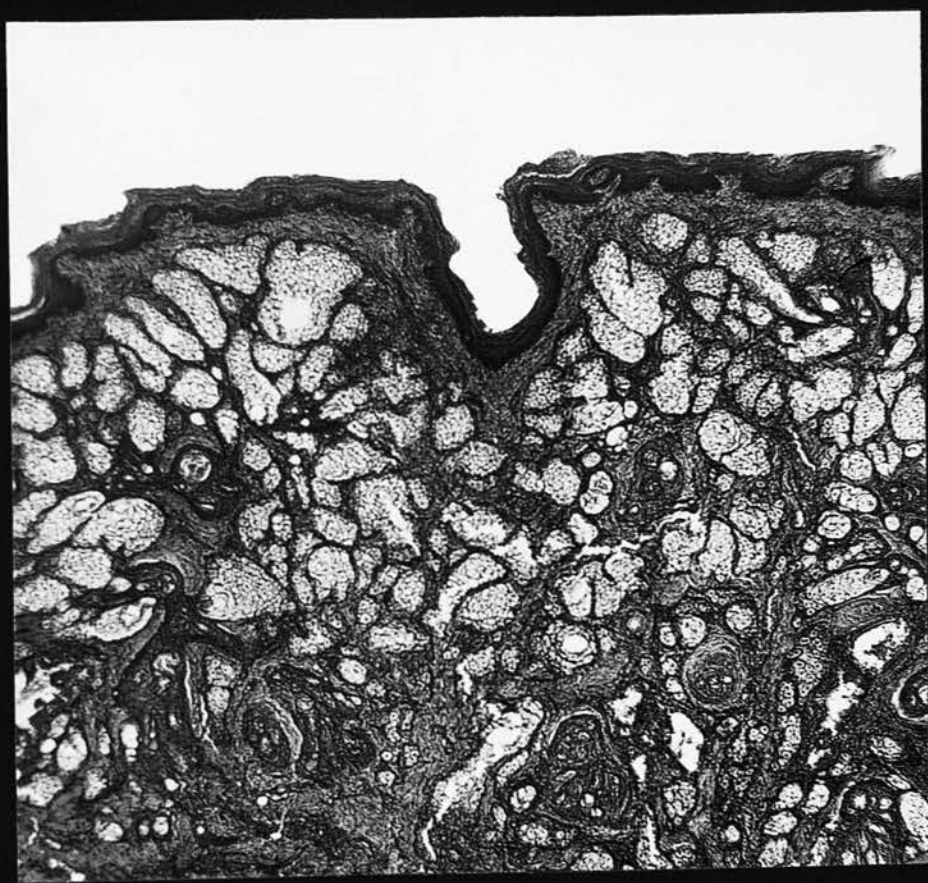
The position of the hair follicles in relation to the hyperplastic glandular tissue.

Hyperplastic connective tissues.

The absence of ulceration and other superficial lesions.

The absence of ceruminous glands which are usually prominent in chronic otitis.

Enlargement x 50



evidence of rete peg formation.

In this type of tumour pockets of hyperplastic glandular material infiltrated the connective tissue of the hypodermis.

PART V - SUMMARY:

Histological preparations of different parts of the external ears of both healthy and affected dogs were examined for evidence of tissue changes which might be correlated with the nature of the discharge and the bacterial findings.

Miller and Witter (1942) attempted to differentiate various clinical forms of otitis histopathologically and in doing so they drew attention to the presence of apocrine tubular glands which they considered to be different from true ceruminous glands.

It was found, in this present work, that these 'modified ceruminous glands' were occasionally present in the deeper layers of the corium of healthy ears whereas, in affected ears, they were not only more numerous but were considerably enlarged, forming enormous cyst-like structures many of which lay either immediately below the epithelium or at the level of the sebaceous glands which tended to be displaced into the hypodermis.

The modified ceruminous glands usually reached the surface of the skin directly or in close proximity to the neck of a hair follicle and only rarely did they join the hair follicle deeper down, as was the usual case with sebaceous glands. Whereas the sebaceous glands united to form a common excretory channel, the so-called ceruminous glands appeared to have individual excretory ducts.

The sebaceous glands of healthy ears differed considerably from those of infected ears. In the former, they were large and

and numerous, whereas in the latter they were frequently much reduced, both in size and activity, and were generally to be found in the deeper layers of the dermis.

It would appear from these findings that the discharges in the infected ears are related to an increase in size and activity of of the tubular apocrine glands and to a corresponding decrease in the number and size of the sebaceous follicles. These changes were particularly noticeable in ears infected with Ps. aeruginosa or Pr. mirabilis which are characterised by a copious, moist, purulent type of discharge.

Conversely, the ears infected only with staphylococci and Pityrosporum species showed little alteration in the size, activity or disposition of the secretory glands. It will be recalled that in this type of otitis the discharge, although abundant and of a darker colour, frequently retains its waxy consistency.

Fibroplasia was also a common feature of affected ears, being especially prominent in the ears of dogs with the more chronic type of otitis. In a number of cases this was sufficiently advanced to give rise to tumour-like swellings of the subcutaneous tissues with a corresponding narrowing of the lumen of the external acoustic meatus.

The most constant feature of all the affected ears was the presence of hyperkeratinization of the surface epithelium with shedding of the surface cells of the stratum corneum. This

This hyperkeratinisation frequently involved the epithelium of the hair follicles which, in dogs, were to be found throughout the length of the external meatus.

Ulceration of the ear canal was less common being confined to some of the more severely affected ears, especially those infected with Gram negative bacteria.

Although adult dogs are only very occasionally affected with otitis media, it is interesting to observe that the cases encountered in this survey were all secondary to an external otitis and that the route of infection appeared to be by way of the tympanum which showed either ulceration or, as in two cases, perforation of the membrane.

Of the invading organisms, the staphylococci and the Pityrosporum species were confined to the superficial layers of the lining epithelium, whereas the Gram negative rods were usually to be seen within isolated pockets of inflammatory cells in the thickened dermis.

Other features observed were the association of tumour formation and chronic otitis, and the role of the tissue mast cells in infected ears.

PART VI.

A detailed study of the more frequently occurring micro-organisms in canine otitis.

CONTENTS.

a) PSEUDOMONAS

The difficulties in classifying the many species in this genus are several and Burkholder and Starr (1948), suggest that there may be only a few true species, but a great number of 'formae speciales' within the group. They also point out that the weakness in the existing classification may be due to failure to recognise which bacteriological features are generic and which are specific.

Gabby (1955) agrees that the genus contains many ill-defined species and suggests that at least some of the characters should be reconsidered.

The species of most importance, in canine otitis, is undoubtedly Ps. aeruginosa which was first isolated by Gessard in 1882 from 'blue pus'. As its former name (Ps. pyocyanea) implies, this organism was frequently identified by its ability to produce a water and chloroform soluble pigment called pyocyanin. It is now recognised that the ability of a strain to produce pigment is frequently lost during subcultivation and Gaby (1946) describes atypical aeruginosa strains which failed to produce pyocyanin.

As many pyocyanin producing strains often form another water soluble pigment called fluorescin, it is frequently difficult to distinguish between Ps. aeruginosa, Ps. fluorescens and other pigment producing bacteria. The work of Seleen and Stark (1943) is of value, in this respect, as they showed that Ps. aeruginosa

Ps. aeruginosa grows well at 42°C. in contrast to other fluorescin producing bacteria which grow but poorly, if at all, under these conditions.

In this present work the genus Pseudomonas will include aerobic, Gram negative, non sporing, actively motile rods which frequently produce a water soluble pigment which diffuses readily throughout the medium. Fermentation of carbohydrates is never pronounced although acid only may be produced from a limited number of sugars.

During the course of this survey 124 strains of Pseudomonas species were isolated from various tissues of dogs affected with otitis, but in the detailed discussion which follows, only the first 90 strains will be considered, all of which were recovered from infected outer or middle ears.

Morphology:

All strains conformed to the above definition in that they were actively motile, non sporing, Gram negative rods, the length of which was subject to considerable variation.

Cultural characters:

All grew well in the usual laboratory media at 37°C, at 27°C and at room temperature. Most strains produced at least one water soluble pigment, in the presence of free oxygen, which readily diffused throughout the medium. On nutrient agar the surface growth was profuse, flat and dry or raised and mucoid

mucoid with a regular, irregular or filamentous outline. In a number of cases the surface of the growth developed a beaten copper or iridescent surface sheen, while most cultures gave off a characteristic odour of trimethylamine. Broth cultures were characterised by abundant turbidity, a heavy granular deposit and frequently a thin surface ring or pellicle. Most strains haemolysed horse blood agar plates and were actively proteolytic.

Metabolic and Biochemical Characters:

The production of Catalase:

The presence of catalase was demonstrated by the production of bubbles when a few drops of 10 vol. Hydrogen peroxide were run over the surface of an overnight nutrient agar slope culture. All strains were active catalase producers.

The reduction of Methylene Blue Milk:

The inoculated media were incubated at 37°C. when coagulation occurred within 48 hours and all strains showed active reduction of the Methylene Blue within 18 hours.

The production of Hydrogen Sulphide:

None of the 90 strains produced H_2S when tested in fluid cultures with lead acetate filter-paper strips or when stab inoculated in lead acetate agar at 37°C. for 7 days.

The Methyl Red and Voges-Proskauer Reactions:

None of the strains showed a final pH of 4.5 or less when

when grown in glucose-phosphate media for 5 days at 37°C., nor was acetylmethylcarbinol produced when tested by the method of Barritt (1936).

Citrate Utilisation:

The ability of Ps. aeruginosa to utilise citrate as the sole source of carbon was tested in Koser's medium. All strains grew profusely except for two which grew very poorly, and then only after 48 hours incubation.

Nitrate Reduction:

The reduction of nitrates to nitrites was tested by the Greiss-Ilosvay method. (Kauffman, 1954).

Kelser and Schoenig (1943) are of the opinion that Ps. aeruginosa reduces nitrates to nitrites. Although Bergey (1948) states that nitrates are reduced to nitrites and nitrogen, Topley and Wilson (1955) point out that the usual nitrate reduction test is negative with most strains after 5 days because the nitrite itself is reduced to gaseous nitrogen. Nevertheless, it is interesting to note that Liu (1952) found that 26 of his 45 strains of Ps. aeruginosa produced nitrite.

In this work the 90 dog strains were tested after 2 and 5 days when only one culture showed a trace of nitrite after 48 hours' incubation.

Indole production: /

Indole production:

Although the formation of indole by Ps. aeruginosa has been reported by various authors, this may be due to the false reactions which result from the action of the acid in Bohme's reagent on the pigment produced by these organisms.

None of our 90 otitis strains were found to produce indole in peptone water cultures which had been incubated at 37°C., and tested after 2 and 5 days with Ehrlich's Rosindole reagent and the ether extraction method.

The Hydrolysis of Urea:

Each strain was tested for its ability to decompose urea on Christensen's agar slopes, the inoculated media being incubated at 37°C. for 6 days. Although strains of Pseudomonas do not hydrolyse urea as promptly as do species of Proteus, 78 (87 per cent) dog strains of Pseudomonas produced a pale pink colouration of the media after 18 hours, 11 (1 per cent) only after 3 or 4 days, while the remaining strain failed to split urea.

The Liquefaction of solidified bovine serum:

The proteolytic activity of Ps. aeruginosa was studied on Solidified bovine serum slopes and in gelatin media. Only 2 of the 90 strains failed to liquefy the surface of solidified serum medium within two weeks, although the great majority did so within 48 hours.

The Liquefaction of gelatin:

Although most aeruginosa strains are actively proteolytic, the ability of a strain to liquefy gelatin is probably of little diagnostic value as the same effect is obtained with other members of the genus.

When the 90 dog strains in this work were stab inoculated into gelatin and incubated at room temperature, the medium was liquefied within 48 hours by 83 strains, within 4 days by 5 strains and within a week by the remaining 2 strains.

Their proteolytic activities in inspissated bovine serum and gelatin media are summarised in the following Table.

TABLE 67

The action of Pseudomonas on inspissated serum and gelatin media

Medium	Number of strains examined	Time in days to produce evidence of liquefaction							Number of proteolytic strains
		1	2	3	4	5	6	7	
Gelatin	90	59	24	4	1	-	1	1	90
Solid serum	90	35	29	11	13	-	-	-	88

The Production of Levan:

Cooper and Preston (1935) and Crosse (1953) have shown that many bacteria synthesise polysaccharides from sucrose which are of the fructosan type and are analogous to the levans formed by B. mesentericus and B. subtilis. Levans are also produced on raffinose but not when the organisms are grown on maltose, lactose, glucose, xylose or fructose (Anderson, 1948). The ability of Pseudomonas to synthesise polysaccharides has been known for some time, this characteristic being readily recognised by the production of large mucoid colonies on sucrose agar.

Paton (1956) has shown that the majority of levan positive strains of Pseudomonas were from plants, a high percentage of which were plant pathogens. On sucrose agar when incubation was prolonged beyond 2 days, colonies of these organisms lost all rigidity, spread over the surface of the agar and dropped on to the cover of the petri dish.

In this present investigation the 90 dog strains were surface plated on nutrient agar containing 5 per cent sucrose so as to give discrete colonies after 2 days' incubation at 27°C.

Although it was found that 39 strains gave moist colonies, none of them showed the characteristics raised 'gummy' growth in the presence of sucrose, nor was there any evidence of loss of rigidity. Moreover, very similar colonies were produced when the strains were grown on plates of nutrient agar and of maltose agar. This suggests that, in contrast to many plant strains,

strains, Pseudomonas species from lesions in dogs do not produce levan.

The haemolytic activity:

Although Haynes (1951) suggests that the characteristic ability of Ps. aeruginosa to haemolyse red cells is of limited usefulness, in the absence of information to show that other members of the genus which grow at 37°C. fail to haemolyse blood, Paton (1956) showed that of 200 strains from plants and soil, only 20 haemolysed 5 per cent bovine blood agar after 4 days at 27°C. All of them were non pathogenic and grew at 37°C. but not at 42°C.

In view of these findings, it was decided to investigate the haemolytic properties of the 90 dog strains all of which were recovered from infected ears and, therefore, potentially pathogenic and were able to grow not only at 37°C. but also at 41°C ± 1. (vide infra.)

Two methods were adopted, the first of which was to streak the cultures on the surface of 5 per cent horse blood agar plates which were incubated at 37°C. and checked for haemolysis after 36 hours.

In the second method the same strains were tested by Kauffmann's (1955) method by which 0.2 ml. of a thrice-washed suspension of horse erythrocytes in saline was added to 2.0 ml. of a 1.0 per cent peptone solution in a narrow tube. This mixture was inoculated by a fine needle point from an overnight

overnight nutrient agar culture, incubated at 37°C. and read at the 36th hour. Of the 90 strains examined, the number showing a haemolytic effect by the two different methods was as follows:-

Method 1: 76 (84 per cent) strains lysed horse red cells within 36 hours

Method 2: 65 (72 per cent) strains gave complete haemolysis after 36 hours

It can be said, therefore, that the majority of Pseudomonas strains of canine origin haemolyse mammalian erythrocytes in contrast to soil and plant strains which only rarely produce a haemolysin.

Colonial Variation:

The presence of colonial variation within the strain was suggested by the fact that a number of Pseudomonas cultures when streaked on the surface of nutrient agar plates produced colonies which varied in size, shape and consistency. The commonest forms were as follows:-

Type (a): Dry and flat with an irregular margin

Type (b): Dry and flat with a filamentous margin

Type (c): Mucoid and raised with a regular margin

Type (d): Mucoid and raised with an irregular margin

The majority of the colonies of types (a) and (b) showed a beaten copper iridescent surface sheen which developed in from 24 to 48 hours. This effect was but rarely produced by the more mucoid types of growth.

Most cultures gave off a characteristic odour of trimethylamine which was most pronounced from the dry iridescent types of growth.

Although many cultures produced colonies of one particular type, it was not uncommon to find at least 2 types of colony in the one culture. These characters are summarised in the following Table.

TABLE 68

The growth characteristics of Pseudomonas aeruginosa on plates of nutrient agar

Number of Strains	Regular	Irregular	Dry	Mucoid	With an iridescent sheen
1	+	+	+	+	+
10	+	+	+	-	+
6	+	+	+	-	-
1	+	-	+	+	+
10	+	-	+	-	+
9	+	-	+	-	-
15	-	+	+	-	+
8	-	+	+	-	-
9	-	+	-	+	-
2	+	+	-	+	+
2	-	+	-	+	+
1	+	+	-	+	-
14	+	-	-	+	-
2	+	-	-	+	+
90	56	54	60	32	43

The results in Table 68 show that of the 90 cultures examined regular colonies were produced by 56 strains and irregular colonies by 54 strains. In addition 20 (22 per cent) strains produced both regular and irregular colonies. A predominantly dry, flat type of growth was produced by 60 strains which is almost twice the number of those that gave a more raised mucoid growth. In only 2 cases were cultures obtained which showed both of these types to be equally prevalent.

Of the 43 cultures which produced colonies with an iridescent surface sheen, 37 were dry and flat, whereas the colonies of 8 strains were raised and mucoid. The presence or absence of a filamentous margin was not related to the iridescent sheen.

Dry growths were as often regular as irregular in outline but the mucoid colonies had usually regular margins.

Pigment Production:

Many members of the genus Pseudomonas are capable of producing at least one water soluble pigment, the commonest of which is fluorescin. Ps. aeruginosa, on the other hand, may produce another pigment, pyocyanin, which is water and chloroform soluble, the presence of which is generally said to be diagnostically significant. The ability of a strain to produce either or both of these pigments is not invariable and many strains lose this power soon after primary isolation. Liu (1952) and Ringen and Drake (1952), refer to a third pigment called pyorubin which is reddish-brown in colour and resembles fluorescin in that it is

is soluble in water but not in chloroform.

Whereas fluorescin may be produced by Ps. aeruginosa, Ps. fluorescens and many other species, pyocyanin is produced only by the first named although a-pyocyanogenic strains are relatively common.

a) Pyocyanin:

The presence of this pigment which is a most important differential feature of Ps. aeruginosa was distinguished by its typical blue-green colour in both solid and fluid media.

This pigment was identified routinely by inoculating nutrient agar slopes and incubating them overnight at 37°C. when they were allowed to stand at room temperature for a further period of 3 days. Portions of the medium and the surface growth were emulsified in a minimal amount of water, shaken up with chloroform and the pigment separated off with a few drops of dilute hydrochloric acid. Of the 90 strains examined by these methods, 55 (61 per cent) were shown to be capable of producing pyocyanin. Although only about two thirds of the dog strains produced pyocyanin, this does not imply that they are not Ps. aeruginosa nor does it suggest that dog strains differ from human or other animal pathogens in their ability to produce this pigment.

As it is obvious from these results that the identification of Ps. aeruginosa should not be based solely on the presence or absence of pyocyanin, it will be necessary to consider the significance of certain other features.

(b) Fluorescin:

The ability of a strain to produce fluorescin is of little diagnostic value except that it frequently masks the presence of pyocyanin in a weak pigment-producing strain of aeruginosa.

With few exceptions the presence of fluorescin was easily recognised on most laboratory media. Nevertheless, in order to detect traces of pigment each strain was cultured on nutrient agar, maltose agar and sucrose agar at 37°C. for 48 hours, followed by a similar period at room temperature, when the presence of fluorescin was confirmed by exposing the plates to a source of ultra-violet light. (The lamp that was used in this experiment was a 'Hanovia' model, Mark II, of 230 volts with a total wattage of 125).

Of the 90 strains examined, 77 (86 per cent) produced fluorescin alone or together with pyocyanin. Only 7 strains failed to produce either pigment but as they grew well at 42°C., they are probably a-pyocyanogenic strains of Ps. aeruginosa.

These findings are summarised briefly in Table 69.

TABLE 69

Pigment production by strains of Pseudomonas of canine origin

Number of strains	Pigment produced: Pyocyanin	Pigment produced: Fluorescin
49	+	+
6	+	-
28	-	+
7	-	-
90	55	77

NOTE: Old cultures of 25 of the 55 pyocyanin producing strains turned reddish-brown in colour, simulating pyorubin, due to oxidation of the pyocyanin.
Pigment was never formed in the absence of oxygen.

Growth at 42°C.

Many authors have tried to establish a satisfactory method for the identification and differentiation of the members of the genus Pseudomonas, and the two important species in particular, viz: Ps. aeruginosa and Ps. fluorescens.

Jordan (1899) has suggested that the latter is a degenerate form, or at least a modified form of the former and that they may be differentiated by their optimum temperature for growth. Unfortunately, it is now known that, although Ps. aeruginosa grows well at 37°C. and Ps. fluorescens does not, there are a

a number of other species which will also grow at 37°C.

Seleen and Stark (1943) have considered this problem and are of the opinion that the good growth obtained at 42°C. with known cultures of Ps. aeruginosa is a feature which, by itself, is sufficient to eliminate all other fluorescent bacteria. These findings have since been accepted by a number of equally reliable workers.

As the dog strains have been shown to be very variable in their haemolytic properties and in their ability to produce detectable amounts of pyocyanin, it was thought necessary to ascertainmhow many strains were able to grow well at 42°C.

For this purpose overnight nutrient agar cultures were inoculated into each of two tubes containing peptone water media by means of a fine needle point. One tube of each pair was incubated overnight at 37°C. and the other was incubated in a carefully adjusted water bath for 24 hours at 41°C. $\pm$ 1.

Of the 90 strains thus examined 76 grew well, 13 grew moderately well, and 1 strain failed to grow. This strain was unusual in that, although it did not grow at 42°C and was non haemolytic to horse erythrocytes, it nevertheless produced both pyocyanin and fluorescin. As pyocyanin is produced only by Ps. aeruginosa, this strain must be accepted as being an atypical aeruginosa.

Carbohydrate fermentation reactions:

There are a number of references in the literature to acid formation from sugars by different species of Pseudomonas. De Bord (1923) reported that in 2 per cent peptone broth containing 1 per cent glucose, all of the glucose could be utilised without producing, at any time, an acid reaction. Sears (1916) showed that Pseudomonas actively metabolised nitrogenous substances even in the presence of glucose while Sears and Gourlay (1928) noted that the acid products of glucose decomposition were neutralised by the products of nitrogen metabolism. Moreover, when other sugars were used in place of glucose, an acid reaction was not produced even when the nitrogen content was low, despite the fact that the sugars tested were said to be capable of being utilised by the organisms.

On the other hand, Lacey (1932) found that, of his Pseudomonas strains, acid was produced in glucose by 90 per cent, in sucrose by 68 per cent but never in lactose. A similar investigation was carried out by Clara (1934) who found that both Ps. aeruginosa and Ps. fluorescens fermented all the monosaccharides, except rhamnose, and that none of them fermented lactose and maltose. Elrod and Braun (1942) examined the fermentative activities of a number of aeruginosa strains and showed that acid only was produced in glucose, xylose and arabinose but not in sucrose, mannitol, maltose, glycerol, salicin or raffinose. These results were largely confirmed by Salvin

Salvin and Lewis (1946).

When agar slants were used instead of the usual fluid media, Ringen and Drake (1952) observed that strains of Ps. aeruginosa, from various sources, produced acid from glucose, galactose, mannose, arabinose and xylose.

Liu (1952) showed that the total amount of alkali produced by Ps. aeruginosa was only a little more than that produced under similar conditions by E. coli or Aerob. aerogenes and that the failure of Pseudomonas to show acid change was due to the small amounts of acid produced from carbohydrates. In order to overcome the masking effect of the alkali produced by preference from the nitrogenous substances in the usual sugar media, Liu (1952) devised a nitrogen free basal synthetic medium to which the required sugar was added when needed. The presence of growth in this medium would indicate that the organism was able to utilise the added carbohydrate and any acid produced could be demonstrated by the addition of a suitable indicator. All of the 45 strains of Ps. aeruginosa which he studied by this method utilised glucose, fructose, mannitol, trehalose and glycerol as a sole source of carbon. They also produced acid from these sugars as they did from arabinose, xylose and mannose although these latter carbohydrates were not utilised as a sole source of carbon. None of the strains acted on dextrin, adonitol, dulcitol, inositol, inulin, lactose, maltose, raffinose, salicin, sorbitol, starch or saccharose.

It would appear, therefore, that the fermentative activities of Pseudomonas depends on the constituents of the media and on the brand of peptone used. The final concentration of the fermentable substance is also of importance, as is the nature and concentration of the nitrogenous base.

In the early part of this present work the first 25 strains of Pseudomonas were tested in ordinary 1per cent peptone water sugars. These were incubated at 37°C. for 14 days and then at room temperature for a further period of 7 days. Because of the intensity of pigment production in certain sugars, especially mannitol and glycerol, which tended to obscure the traces of acid that appeared irregularly in a number of sugars after 7 to 10 days' incubation, the need to devise a better method of studying the sugar reactions of Pseudomonas soon became apparent. To this end the same 25 strains were tested by Liu's methods, 1 per cent of the required carbohydrate being added as required to a previously prepared synthetic medium of the following composition:-

$(NH_4)_2.SO_4$	2.0 gm
$MgSO_4$	0.2 gm
$CaCl_2$	0.1 gm
NaCl	0.2 gm
K_2HPO_4	0.2 gm
Phenol Red (0.2%)	8.0 ml
Distilled water	1000 ml

pH 7.2

The inoculated sugars were incubated at 37°C. for 4 days when they were examined for the presence of growth and acid formation which was shown by the light pink colour of the medium changing to a deep canary yellow.

The results thus obtained are summarised in the following Table.

TABLE 70

The fermentation of carbohydrates by Pseudomonas

Medium	Number of strains examined	Glucose	Lactose	Mannitol	Sucrose	Salicin	Arabinose	Trehalose	Xylose	Rhamnose	Glycerol
Peptone water sugars	25	22	0	7	0	0	18	0	25	0	4
Synthetic medium sugars	25	25	0	23	5	0	24	15	25	0	25

Owing to the fact that the acid and point was much more obvious in the synthetic sugar medium, pigment production being rarely visible, it was decided to retain this method for the other strains as the results obtained were almost identical to those of Liu and other contemporary workers.

The fermentative activities of the 90 Pseudomonas strains of canine origin are shown in Table 71.

TABLE 71

The utilisation by Pseudomonas of carbohydrates in a nitrogen free medium

Number of Strains	Glucose	Lactose	Mannitol	Saccharose	Salicin	Arabinose	Trehalose	Xylose	Rhamnose	Glycerol	Galactose	Mannose	Laevulose
47	+	-	+	-	-	+	-	+	-	+	+	+	+
25	+	-	+	-	-	+	+	+	-	+	+	+	+
6	+	-	+	+	-	+	-	+	-	+	+	+	+
4	+	-	+	+	-	+	+	+	-	+	+	+	+
1	+	-	-	+	-	+	+	+	-	+	+	+	+
3	+	-	-	-	-	+	+	+	-	+	+	+	+
2	+	-	-	-	-	+	-	+	-	+	+	+	+
1	+	-	+	-	-	-	+	+	-	+	+	+	+
1	+	-	+	-	-	+	-	-	-	+	+	+	+
90	90	0	84	11	0	89	34	89	0	90	90	90	90

These results show that, with the exception of two strains, one of which failed to ferment arabinose and the other xylose, all Pseudomonas strains of canine origin produced acid but not gas in glucose, arabinose, xylose, glycerol, galactose, mannose

mannose and laevulose. Most of the strains (93 per cent) attacked mannitol, a number (38 per cent) trehalose and a few (12 per cent) saccharose. None of the strains fermented lactose, salicin or rhamnose. It is also interesting to notice that primary cultures of 76 (84 per cent) of the same 90 strains showed a trace of acid only when tested in 1 per cent glucose peptone-water media.

The identification of Ps. aeruginosa:

Although the Pseudomonas strains of canine origin have been submitted to a number of important tests, no attempt has been made to distinguish different species. While it is admitted that the ability of a strain to produce pyocyanin is a diagnostic feature of Ps. aeruginosa, there are numerous references, in the literature, to a-pyacyanogenic variants. Some workers claim that a more reliable criterion is the ability of Ps. aeruginosa to grow at 42°C. while others stress their haemolytic properties. During this work it was thought that the iridescent beaten copper sheen on the surface of agar cultures might be of some importance and it is proposed that this be included with the three previously mentioned characteristics in an attempt to ascertain their significance as diagnostic features of Ps. aeruginosa.

TABLE 72

A comparison of four important characteristics of *Pseudomonas*

Number of strains	Pyocyanin produced	Growth at 42°C	Haemolysis of horse red cells	Iridescent sheen on surface colony
29	+	+	+	+
17	+	+	+	-
7	+	+	-	+
1	+	+	-	-
1	+	-	-	+
4	-	+	+	+
15	-	+	+	-
14	-	+	-	-
2	-	+	-	+
90	55	89	65	43

If pyocyanin is produced only by *Ps. aeruginosa* then 55 (61 per cent) of the 90 dog strains are of this type. Of the 55 pyocyanin producers all but one grew at 42°C., 46 (84 per cent) were haemolytic and 37 (67 per cent) produced iridescent colonies. While all of the 35 a-pyocyanogenic strains grew at 42°C., 19 (54 per cent) were haemolytic but only 6 (17 per cent) showed iridescent colonies on nutrient agar. It is probably true to say that as all 35 non-pyocyanin producing strains grew at 42°C., the absence of pigment is due rather to

to strain variation than to the fact that they are not Ps. aeruginosa.

It will also be recalled that the low incidence of pigment producing strains is due to the unsuitability of routine media for this purpose, although Haynes (1951) found that approximately 20 per cent of his aeruginosa cultures failed to produce pyocyanin when grown on selective media.

Although 65 (72 per cent) dog strains showed a haemolytic effect on horse red cells, its value as a diagnostic aid is doubtful as Paton (1956) has found that a number of plant pathogens, other than Ps. aeruginosa, showed haemolytic properties. It will also be noticed that 10 per cent of the pyocyanin-producing dog strains failed to haemolyse horse blood.

In the same way, the presence of iridescent plaques on nutrient agar cultures is not diagnostically significant, nor is it directly related to pigment production, as the colonies of 18 (20 per cent) pyocyanin-producing strains were non-iridescent.

In recent years a number of workers including Seleen and Stark (1943), Haynes (1951), Ringen and Drake (1952) and Liu (1952) have expressed the opinion that the ability to grow at 42°C. is a reliable guide to the identity of Ps. aeruginosa, whether or not pyocyanin is produced. As the strains under discussion were all recovered from lesions, and either grew at 42°C. or produced detectable amounts of pyocyanin, it is perhaps permissible to classify them as Ps. aeruginosa.

On this assumption it is interesting to compare their sugar reactions with those of other workers who studied known strains of aeruginosa.

	Glucose	Galactose	Mannose	Arabinose	Xylose	Glycerol	Trehalose	Mannitol	Laevulose	Sucrose
≠ Moltke (1927)	-	-	-	-	-	-	-	-	-	-
≠ Bergey (1948)	+	-	-	-	-	-	-	-	-	-
Elrod and Braun (1942)	+	.	.	+	+	-	.	-	.	-
Gaby (1946)	+	+	.	.	+	+	.	.	.	.
Salvin and Lewis (1946)	+	+	+	+	+	+	-	-	-	-
Ringen and Drake (1952)	+	+	+	+	+	.	.	.	.	.
Liu (1952)	+	+	+	+	+	+	V	+	+	-
Our results	+	+	+	+	+	+	V	±	+	∓

≠ = In peptone water sugars
\+ = Acid only
\- = No acid produced
. = Results not stated
V = Results variable
± = Acid produced by most strains
∓ = Acid produced by an occasional strain

Pathogenicity:

In their discussion of the Straus test for the diagnosis of glanders, Gaiger and Davies (1947) mention that a number of organisms including Ps. aeruginosa may set up an orchitis when

when inoculated intraperitoneally into male guinea pigs. In order to confirm this and in an attempt to ascertain the property of the strain that is responsible for its pathogenicity, thirty selected strains of Ps. aeruginosa from infected ears were examined biologically.

The strains were grown overnight in broth at 37°C. when 0.5 ml. was inoculated intraperitoneally into a young male guinea pig of approximately 200 kilos body weight. The animals which were examined daily were slaughtered on the 10th day.

The results obtained are summarised in the following Table.

TABLE 73

The pathogenicity of Ps. aeruginosa to young male guinea pigs

Number of strains	Pigments produced	Number of strains producing - Death	Orchitis	Abscesses	No Reaction
3	P.	0	1	0	2
15	P.F.	1	4	1	9
11	F.	0	1	6	6
1	A.	0	0	0	1
30		1	6	7	18

P. = Pyocyanin only
P.F. = Pyocyanin and Fluorescin
F. = Fluorescin only
A. = Achromogenic strain

The results of this experiment were very disappointing in that only 5 of the 30 aeruginosa strains produced a characteristic orchitis. It will be noticed that 5 out of 18 pyocyanin producers and only 1 out of 11 a-pyocyanogenic strains gave rise to a typical Straus reaction. Of the 7 strains producing other lesions, 5 strains resulted in the formation of abscesses within the peritoneal cavity and 2 strains gave rise to a single large circumscribed abscess on the surface of one testicle of each of the 2 affected guinea pigs.

As no fewer than 11 out of 18 pyocyanin-producing strains of Ps. aeruginosa failed to produce lesions in guinea pigs, the experiment was abandoned.

Phage typing:

By phage typing methods, 41 Ps. aeruginosa strains of canine origin were divided into 7 main types, as is shown in Table 74.

TABLE 74

The phage types of Ps. aeruginosa from infected canine ears

Phage type	Number of strains
J.	17
GK.	6
W.	4
Y	3
CQS.	1
QS.	2
Q.	1
no type	4
no result	1
miscellaneous	2
Total	41

NOTE: The so-called 'type', denoted by a letter or group of letters, refers to the leading phage filtrate of a group of phages which have reacted with the organism in question.

Although similarity of type is not absolute proof of identity, it will be seen from the results in Table 74 that many of the dog strains are similar in pattern to those obtained from human sources.

TABLE 75.

The characters of Ps. aeruginosa of canine origin.

Character	No. of strains giving a positive reaction.	
Motility	90	
Indole production	0	
Nitrate reduction	1	
H_2S production	0	
Gelatin liquefaction	90	
Serum digested	88	
Catalase production	90	
Citrate utilisation	90	
Growth in hydrogen	1	
Growth at 42°C.	89	
Haemolysis (Kauffman)	65	
Methylene-blue reduction	90	
Urea hydrolysis	89	
Levan production (Sucrose agar)	0	
Methyl Red reaction	0	
Voges Proskauer reaction	0	
Pyocyanin production	55	
Fluorescin production	77	
Colonial characters:		
Regular	56	
Irregular	54	
Dry	60	
Moist	32	
Iridescent	43	
Odour of trimethylamine	81	
Acid from:	A.	B.
Glucose	100 %	88 %
Lactose	0	0
Mannitol	93	28
Sucrose	12	0
Salicin	0	0
Arabinose	99	72
Trehalose	38	0
Xylose	99	100
Rhamnose	0	0
Glycerol	100	16
Galactose	100	-
Mannose	100	-
Laevulose	100	-

Note: The 90 strains in column A. were tested in nitrogen-free sugar media.
The 25 strains in column B. were tested in peptone water sugar media.

PART VI.a. - SUMMARY:

Ninety strains of Pseudomonas from infected dogs' ears were examined in some detail.

In general terms they were aerobic, non-sporing, actively motile, Gram-negative, pleomorphic rods which were M.R. and V.P. negative and which failed to reduce nitrates to nitrites or produce indole and hydrogen sulphide. Gelatin, solidified bovine serum and urea were hydrolysed, methylene-blue milk was reduced, citrate was utilised, catalase was produced but none of the strains formed levan on sucrose agar. A number of different colonial forms were seen on nutrient agar plates and most strains produced either, or both, of the water soluble pigments pyocyanin and fluorescin. The majority of strains grew well at 42°C., gave off a characteristic odour of trimethylamine and haemolysed horse erythrocytes. In nitrogen free sugar media most strains produced acid only from glucose, arabinose, glycerol, galactose, mannose, laevulose and mannitol. The reaction in trehalose was variable and very few strains attacked saccharose. None of them fermented lactose, salicin or rhamnose. Although all of the strains were recovered from lesions and were subsequently shown to be Ps. aeruginosa, approximately 40 per cent failed to produce pyocyanin on the usual laboratory media.

It is also suggested that the active proteolytic and haemolytic properties of these strains are not diagnostically significant as they are probably shared by other members of the

the genus.

A few strains gave rise to an orchitis when inoculated intraperitoneally into male guinea pigs and, although most of these were pyocyanogenic, the great majority of pyocyanin-producing strains failed to produce lesions within ten days.

b) PROTEUS

In this paper the genus Proteus was defined as motile rods, conforming to Kauffmann's (1954) definition of Enterobacteriaceae, which usually fermented glucose, glycerol and xylose, frequently sucrose and salicin, but never lactose or dulcitol. All strains reduced nitrates, decomposed urea, and usually liquefied gelatin. The genus was subdivided into four species in accordance with the recommendations of Rustigan and Stuart (1945), namely Pr. vulgaris, Pr. mirabilis, Pr. morganii and Pr. rettgeri.

Source of Strains:

During the various investigations of the flora of normal and affected dogs, a total of 182 Proteus strains were recovered. These were provisionally identified as Pr. mirabilis 172 strains, Pr. vulgaris 9 strains and Pr. morganii 1 strain. There was no evidence of Pr. rettgeri in any of the tissues examined. Unfortunately, it was not possible, for practical reasons, to submit all of these strains to the necessary detailed investigation and so 100,strains were selected for further study. This figure includes the first 90 consecutively occurring strains of Pr. mirabilis, 9 of Pr. vulgaris and the single strain of Pr. morganii. As only 4 of the total of 166 Proteus strains from infected ears were found to be Pr. vulgaris, both tonsil strains and the three rectal strains from healthy dogs have been included to provide a sufficient number for comparison with the

the Pr. mirabilis strains.

Morphology:

Without exception, all strains were found to be Gram negative, non-sporing bacilli, the size of which was subject to great variation. Although many of the strains were sluggishly motile in the original cultures, only 2 strains of the entire collection were found to be non motile at 37°C. and 25°C. This compares favourably with the results of Rustigan and Stuart (1945) who reported only 4 non-motile Pr. mirabilis strains out of the 205 strains examined by them. Lovell (1929) observed that a few of his morganii strains were non-motile on first isolation and that all but one were motile on subcultivation. A similar result was obtained with the single strain of Pr. morganii from otitic material which was non-motile at first but was motile when re-examined some 3 months' later.

Growth:

All grew well at 37°C., 25°C and 17°C. They were all aerobes and facultative anaerobes but the presence of free oxygen in a moist atmosphere was preferred.

Swarming:

Following on Cantu's (1911) observation that, if an organism of the Proteus group were inoculated into the condensation water of an agar slope, a rapid spreading type of growth occurred over the whole surface of the medium, Horowitz (1916) and Moltke (1927)

Moltke (1927) examined this phenomenon more thoroughly. Moltke found that, while swarming and motility were variable, he was able to correlate the presence or absence of motility with the swarming activities of his 197 strains.

Of the dog strains, only 3 failed to swarm on 1.5 per cent nutrient agar of which 2 were non-motile Pr. mirabilis and the third was Pr. morganii .

Urease Test:

The value of the urease test in the identification of Proteus is stressed by a number of workers including Minning and Ritter (1937), Edwards and Ewing (1955), Topley and Wilson (1955) and Kauffmann (1954). As Cook (1948) noted during his investigation of 120 strains, all four species within the genus produced urease. Of these Pr. mirabilis, Pr. vulgaris and Pr. rettgeri show a marked alkalinity in Christensen's urea agar after 6 to 8 hours, whereas Pr. morganii utilised urea more slowly.

Of the 100 strains from dogs, 76 hydrolysed urea within 6 hours, 23 in from 6 to 8 hours and one, the Pr. morganii strain, within 12 hours. There appeared to be no difference in the rate of urease production between the Pr. mirabilis and Pr. vulgaris strains.

Indole Production:

Hauser's original subdivision of the Proteus group into 3 species, on the basis of morphology, rate of liquefaction of

of gelatin and indole production is no longer acceptable as it is now known that the first two of these characteristics are very variable and the last, the production of indole, was formerly tested by the nitroso-indole method which reacts not only to indole but also to indole acetic acid. It is now generally agreed that the presence of indole, as detected by Ehrlich's reagent, is an important distinguishing feature of strains of Pr. vulgaris and Pr. morganii from typical strains of Pr. mirabilis.

Whilst Cantu (1911) drew attention to the fact that indole production is marked in strains of animal origin, later workers have been able to correlate indole production with the fermentation of maltose and mannitol. (Bengston, 1919, Bach, 1921, and Moltke 1927). Because of this, the Proteus strains of canine origin were provisionally identified as Pr. vulgaris, if they produced indole and promptly fermented both maltose and saccharose, Pr. mirabilis, if neither indole was formed nor maltose fermented, and Pr. morganii, if indole was produced in the absence of maltose fermentation and gelatin liquefaction.

Of the 100 strains examined, only 9 produced indole, 8 of which promptly fermented maltose. On the other hand, one indole negative strain attacked both maltose and saccharose to produce acid and gas within 18 hours, whereas maltose was unaffected by the other 90 indole negative strains.

Sugar Fermentation Tests:

The results are summarised in the following Table, the strains being listed in their respective subgroups for the sake of convenience.

TABLE 76

Summary of the sugar reactions of 100 strains of Proteus

Subgroup	No. of Strains	Days of incubation of sugars	Acid and gas in - Glucose	Maltose	Sucrose	Trehalose	Salicin	Galactose	Glycerol	Xylose	Laevulose	Mannose
Pr. mirabilis	90	1 - 4	90	0	29	90	1	89	87	89	48	0
		5 - 21	90	0	73	90	16	89	90	89	88	0
Pr. vulgaris	9	1 - 4	9	9	9	9	6	9	7	9	5	0
		5 - 21	9	9	9	9	7	9	8	9	9	0
Pr. morganii	1	1 - 4	1	0	0	0	0	1	0	0	1	1
		5 - 21	1	0	0	0	0	1	1	0	1	1

None of the strains fermented lactose, mannitol, dulcitol, inositol, raffinose, arabinose, rhamnose, dextrin, inulin or sorbitol.

It will be seen from the results in Table 76 that all strains fermented glucose and also, with one exception, galactose and glycerol. Maltose was attacked only by Pr. vulgaris, and mannose by Pr. morganii. Although sucrose was fermented by all the Pr. vulgaris and by most of the Pr. mirabilis strains, 44 of the

the latter had no effect on this sugar during the first 4 days of incubation, while another 17 failed to act within 21 days. Salicin was promptly fermented by Pr. vulgaris but not by Pr. mirabilis, although 16 strains did so slowly. It is evident from these results that Proteus bacilli of canine origin are very similar in their fermentative activities to those from human and other animal sources.

It is now possible to correlate the sugar reactions of the Proteus strains with their ability to produce indole in peptone water. (See Table 78, page 229).

TABLE 77

Correlation of urease activity, indole production, sugar fermentation and the ability to swarm on nutrient agar

Species	Examined	Giving the reaction cited	Urease	Swarming	Indole	Maltose	Sucrose	Mannose
Pr. mirabilis	9	8	+	+	+	+	+	+
		1	+	+	-	+	+	-
Pr. vulgaris	90	71	+	+	-	-	+	-
		17	+	+	-	-	-	-
		2	+	-	-	-	+	-
Pr. morganii	1	1	(+)	+	+	-	-	+

(+) = Urea slowly hydrolysed

Bengston (1919), was one of the first to notice that all indole positive strains might be expected to ferment both maltose

TABLE 78.

Biochemical (fermentative) types of Proteus.

	Pr. morganii	Pr. mirabilis								Pr. vulgaris			
	(1 strain)	(90 strains)								(9 strains)			
	Type	Type								Type			
	I	I	II	III	IV	V	VI	VII	VIII	I	II	III	IV
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	+	+	+	+
Sucrose	-	x	-	x	x	-	x	x	x	+	+	+	+
Trehalose	-	+	+	+	+	+	+	x	+	x	+	+	+
Salicin	-	-	-	x	-	x	v.	v.	-	+	v.	+	x
Galactose	x	+	+	+	+	+	+	v.	+	+	+	+	+
Glycerol	x	x	x	x	+	x	x	x	x	x	x	-	x
Xylose	-	+	+	+	+	+	+	+	-	+	+	+	+
Laevulose	+	x	x	x	x	x	x	-	x	x	x	x	x
Mannose	+	-	-	-	-	-	-	-	-	-	-	-	-
Total strains	1	50	16	12	4	1	4	2	1	3	4	1	1

Note: No strain fermented lactose, mannitol, dulcitol, inositol, raffinose, arabinose, rhamnose, dextrin, inulin or sorbitol.

Key: + = fermentation within 24 hours.
x = late fermentation, after 24 hours.
v. = irregularly positive or negative.

maltose and sucrose, a finding which has subsequently been confirmed by a number of workers including Bach (1921), Moltke (1927), and Rustigan and Stuart (1945).

One strain from a case of otitis is included in the Pr. vulgaris group, as it not only fermented maltose and sucrose overnight but it also produced both acid and gas in salicin within a week. The only other atypical strains were two of Pr. mirabilis which were non-motile and did not swarm on 1.5 per cent agar.

Proteolytic Activity:

A characteristic feature of the Proteus group of organisms is their activities in gelatin and solidified serum media.

Rustigan and Stuart (1945) examined 78 strains of Pr. rettgeri, and the following workers, between them, examined 264 strains of Pr. morganii, without finding a single strain that hydrolysed gelatin. (Rustigan and Stuart, 1945; Cook, 1948; Jordan, Crawford and McBroom, 1935; and Rauss, 1936). Similar findings with strains of Pr. morganii of animal origin were reported by Lovell (1929) and Phillips (1955).

In contrast to this, a review of the literature shows that all workers are agreed that gelatin is usually liquefied by Pr. mirabilis and Pr. vulgaris. On inspissated serum, however, their proteolytic activity is less marked and this appears to be especially true of the Pr. vulgaris strains.

In this work stab cultures in nutrient gelatin were maintained at room temperature for 3 weeks and in deep inspissated

inspissated bovine serum at 37°C. for 14 days. The results of these investigations are summarised in the following Table:-

TABLE 79

The proteolytic activity of *Proteus* strains of canine origin

Species	Number of strains examined	Incubation Time	Liquefaction of :- Gelatin	Solidified Serum
Pr. mirabilis	90	1 - 3 days	86	50
		4 days or more	3	37
Pr. vulgaris	9	1 - 3 days	8	2
		4 days or more	1	7
Pr. morganii	1	1 - 3 days	0	0
		4 days or more	0	0

These results show that only one of the *Pr. mirabilis* strains failed to liquefy gelatin and that the rest did so within 3 days, except for two strains which required 9 days. Only 2 strains failed to liquefy inspissated bovine serum and 37 strains did so only after 10 to 14 days' incubation.

While the number of *Pr. vulgaris* strains is small, it is interesting to notice that they all liquefied both gelatin and inspissated serum, although the action in serum of the majority of strains was delayed.

The single strain of *Pr. morganii* was typical in that it

it showed no proteolytic activity within 6 weeks.

The Methyl-Red and Voges-Proskauer Reactions:

The presence of acetylmethylcarbinol was determined by Barritt's (1936) method which is probably more sensitive than O'Meara's modification (Topley & Wilson, 1955).

The results of the M.R. tests showed that 81 of the 90 strains of Pr. mirabilis were positive, the remaining 9 strains being described as weak positive. On the other hand, no fewer than 3 of the 9 Pr. vulgaris strains were negative, while the other 6 strains and the strain of Pr. morganii were positive.

The accurate interpretation of the Voges-Proskauer reactions was found to be extremely difficult due to the small and very variable amounts of acetylmethylcarbinol produced. Most workers are of the opinion that Proteus bacilli are rarely, if ever, V.P. positive (Bengston, 1917; Fulton and Harrison, 1943; Minning & Ritter, 1943; and Pandit, 1936), but Rustigan and Stuart (1945) and Cook (1948) have drawn attention to moderate or weak reactions in the case of a number of Pr. mirabilis strains. These latter authors also agree that the same is not the case with Pr. vulgaris, Pr. morganii and Pr. rettgeri.

The M.R. and V.P. reactions of the Proteus strains of canine origin are summarised in Table 80.

TABLE 80

The Methyl-Red and Voges-Proskauer reactions of *Proteus* bacilli

Species	Number examined	Methyl-Red Test		Voges-Proskauer Test (Acetylmethylcarbinol produced)		
		Positive	Negative	Moderate	Trace	None
Pr. mirabilis	90	90+	0	34x	12	44
Pr. vulgaris	9	6	3	1	2	6
Pr. morganii	1	1	0	0	0	1

\+ = includes 9 weak reactions
x = includes 9 very strong reactions

The results in Table 80 show that the *Pr. mirabilis* strains of canine origin were very similar to those of Rustigan and Stuart (1945) who found that 119 of 205 strains were V.P. positive although the reactions were usually moderate and often weak. Cook (1948), on the other hand, reporting that 14 of his 86 *Pr. mirabilis* strains produced acetylmethylcarbinol, qualified his remarks by stating that the reactions were weak and difficult to interpret.

As none of them found V.P. positive strains in the *Pr. vulgaris* group, it is therefore interesting to note that three of our strains were M.R. negative, one of which was strongly V.P. positive, the other two being weakly positive.

Citrate Utilisation:

During his studies on coliform bacilli, Koser (1923) devised a synthetic medium in which citrate was present as the sole source of carbon. The ability of Proteus to utilise the citrate in this medium has been investigated by a number of workers of whom Speck and Stark (1942) attempted to differentiate the species by correlating the M.R., V.P., indole and citrate reactions with the gas ratio and final pH. of 66 freshly isolated Proteus cultures. These, they claim, are more stable characteristics of Proteus organisms than sugar fermentation reactions, especially as regards old stock cultures many of which frequently lose their power to ferment sucrose and maltose.

However, while it is agreed that Pr. morganii does not, and Pr. rettgeri does utilise citrate, the reactions of the more commonly occurring Pr. mirabilis and Pr. vulgaris tend to be rather too variable for Speck and Stark's classification to be of practical value.

In this present work, 85 of the 90 Pr. mirabilis strains utilised sodium citrate although 14 did so only weakly. On the other hand, the single strain of Pr. morganii and approximately half of the Pr. vulgaris strains failed to grow in Koser's medium. This agrees with the findings of Rustigan and Stuart (1945) who reported all 102 Pr. morganii strains as citrate negative, 36 of 69 Pr. vulgaris strains and only 9 of 205 Pr. mirabilis strains as citrate positive.

Hydrogen sulphide production:

It is now agreed that, typically, Pr. mirabilis, Pr. vulgaris and Pr. morganii produce hydrogen sulphide although the ability of the last-named species to do so is not pronounced and is slow to develop. In consequence, Pr. morganii strains have often been described in the literature as non-H_2S producing strains (Kauffmann, 1955), whereas in fact Pr. rettgeri is probably the only species in the genus to fail in this respect.

The Proteus strains of canine origin were tested for hydrogen sulphide production by stab inoculation in lead acetate agar and also by means of lead acetate impregnated filter strips which were suspended over, without actually touching, the surface of nutrient agar slope cultures.

Except for three Pr. mirabilis strains which gave only a trace of hydrogen sulphide, all the other Pr. mirabilis and the Pr. vulgaris strains were strongly positive. The strain of Pr. morganii showed a trace of colour in the lead acetate agar only after 4 days incubation, and is classified as being weakly positive.

Nitrate Reduction:

This was tested by the Greiss-Ilosvay method. A pink red or maroon colour developed immediately in the presence of nitrites.

None of the Pr. mirabilis, Pr. vulgaris or Pr. morganii strains from dogs failed to reduce nitrates to nitrites.

Methylene Blue and Litmus Milks:

The dye in methylene blue milk media was rapidly reduced by all strains. In litmus milk, however, the reactions were somewhat variable as most strains, after an initial and temporary acidity, turned the media markedly alkaline by the 14th day of incubation at 37°C. A rennet clot was usually produced within 48 hours, digestion of which, accompanied by progressive alkalisation of the medium, was invariably complete by the 10th day. The time required for these changes to take place is shown in the following Table.

TABLE 81

The formation and digestion of the clot in Litmus milk by *Proteus* bacilli

Species	Number examined	Incubation time in days	Litmus milk medium: Clot formed	Clot digested
Pr. mirabilis	90	1 - 2	84	0
		3 - 4	3	74
		6 - 10	2	15
Pr. vulgaris	9	1 - 2	3	0
		3 - 4	2	2
		6 - 10	2	5
Pr. morganii	1	1 - 14	Reaction very alkaline	

Haemolysis:

Each strain was tested in turn for its lytic activity by the method of Kauffmann (1955).

It was found that 55 of the 90 Pr. mirabilis strains were actively haemolytic, 18 weakly haemolytic and 17 non haemolytic to horse red cells. All 9 Pr. vulgaris strains were actively haemolytic while the single strain of Pr. morganii had no effect on horse erythrocytes.

Although the number of strains in each group is small, the difference in the haemolytic activities towards horse cells of strains of Pr. mirabilis and Pr. vulgaris of animal origin was similar to Phillip's (1955) findings except that more of our dog strains of Pr. mirabilis were haemolytic when tested by the above methods. In this connection it is interesting to recall that Wenner and Rettger (1919) found that none of the strains examined by them were haemolytic and yet Taylor (1928) showed that all of his strains lysed human erythrocytes.

Catalase production:

All Proteus strains, irrespective of species, were found to be strong catalase producers.

The Dienes' Phenomenon and its application to a study of the source and spread of Proteus bacilli in dogs suffering from otitis

During the examinations of chronic cases of otitis, it was noticed that many of the dogs with Proteus infected ears harboured the same organism in other parts of the body. This suggested that a study of these organisms would probably throw some light on the source and spread of infection to affected ears. In doing so, advantage was taken of the phenomenon described by Dienes (1946) to distinguish antigenically identical strains of Proteus bacilli.

Dienes observed during his investigations of the reproductive processes in Proteus cultures that large bodies were present in small numbers in the spreading halos of most strains. Accidental observations, however, suggested that these large bodies were more numerous in mixed cultures of different strains and, when broth cultures of certain strains were mixed and plated on agar, large bodies appeared in clusters situated at the edge of and between the originally developed colonies when spreading started. This interaction between strains was best seen when strains were plated separately on agar and the zone where the spreading halos met were examined. When two halos met, most of the filaments were transformed into large bodies and were thus immobilised within a short time. Before large bodies were produced in mixed cultures it was necessary for at least two fundamental conditions to be fulfilled namely, the strains must be appropriate and

and spreading halo must meet spreading halo. Filaments spreading from two colonies of the same strain were found to exert no influence on each other when the halos met. Finally, the fact that halos between mixed strains may stop short of each other was taken to indicate diffusion into the agar of antagonistic substances.

This was subsequently investigated by Krikler (1953) who compared a number of Proteus strains in relation to the Dienes phenomenon and the antigenic structure. He found that, with very few exceptions, no line of demarcation appeared between antigenically identical strains, particularly if the flagellar antigens were the same. Conversely, if the antigenic structure was different, a line of demarcation did appear when two strains were allowed to swarm together. He concluded that the Dienes' phenomenon gave more specific results than were obtainable by somatic antigen typing.

These findings suggested a comparatively simple, if laborious, method of accurately determining whether Proteus strains from different sites of the same dog were antigenically identical. It was hoped to show, by examining a sufficient number of cases, that a particular type was present in affected ears which was also to be found in a particular site of the animal's body. If this was so, it might then be possible to demonstrate that the same strain-type predominated in the same site in healthy dogs.

Methods:

Cultures of Proteus which showed a wave-like pattern on the surface of solid media were inoculated into the condensation water of nutrient agar slopes and incubated for 18 hours at 37°C. Phase B and Phase C organisms, or cultures showing variation to either of these phases, were considered to be untypable. (Phase B is usually non-swarming and Phase C swarms in a continuous film). (Belyavin, 1951).

Poured plates containing 2 per cent nutrient agar were dried in the incubator at 37°C. for half an hour to ensure that no moisture remained on the surface of the medium. Each strain was then tested against itself and against two unknown strains by spot inoculation on the four 'corners' of each plate. The plates were incubated at 37°C. overnight when the spreading halos were examined for the presence of lines of demarcation (of no growth), about 2 mm. wide, between two antigenically distinct strains.

Four patterns are possible by this method namely:

(1) Where the test strain and the two unknowns are identical, no line of demarcation is seen.

(2) Where the two unknowns are identical but are distinct from the test strain (Plate 30).

(3) Where the unknowns are dissimilar but one of them is identical to the test strain (Plate 31).

(4) Where the unknowns and the test strains are all antigenically dissimilar (Plate 32).

PLATE 30.

The Dienes' phenomenon.

Key to plate.

Strain 5.	Strain 5.
Strain 29.	Strain 87.

Showing three strains of Pr. mirabilis on the surface of a 1.5 % nutrient agar plate, incubated at 37°C. for 18 hours.

Interpretation. The double inocula of Strain 5, of known antigenic type, are used as a control.

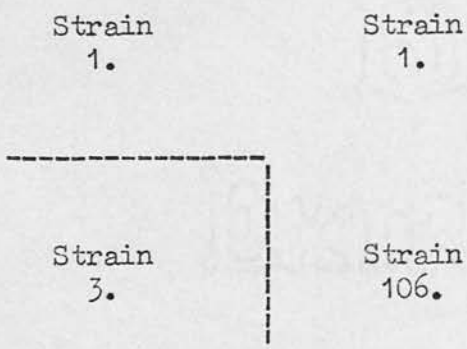
Strain 5 is antigenically dissimilar to both strains 29 and 87.

Strains 29 and 87 are antigenically similar.

PLATE 31.

The Dienes' phenomenon.

Key to plate.



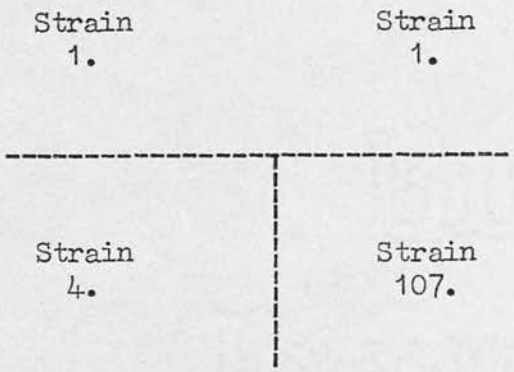
Showing three strains of Pr. mirabilis on the surface of a 1.5 % nutrient agar plate, incubated at 37°C. for 18 hours.

Interpretation. Strains 1 and 106 are antigenically similar but are distinct from Strain 3.

PLATE 32.

The Dienes' phenomenon.

Key to plate.



Showing three strains of Pr. mirabilis on the surface of a 1.5 % nutrient agar plate, incubated at 37°C. for 18 hours.

Interpretation. All three strains (1, 4 and 107) are antigenically dissimilar.

Results:

The first part of this investigation was confined to an examination of 79 strains of Pr. mirabilis which were isolated from infected ears of the dogs in the main survey (See Part II of this work).

It was possible by testing each strain against the other, and against themselves, to subdivide 67 of these strains into 14 distinct groups or types. Four strains were untypable due to an alteration in phase during subcultivation, and the examinations of another 8 strains were discontinued because of variation during the period of the experiment.

TABLE 83

The classification of Pr. mirabilis strains from infected ears into antigenically distinct types

Designation of Group	Number of Strains in each Group	Unilateral otitis	Bilateral otitis	
			I Similar	I Dissimilar
I.	32	14	8	2 + x
II.	3	3	-	-
III.	6	4	1	-
IV.	1	1	-	-
V.	1	1	-	-
VI.	2	1	-	1 x
VII.	1	1	-	-
VIII.	2	-	1	-
IX.	2	-	1	-
X.	3	1	1	-
XI.	2	-	1	-
XII.	6	2	2	-
XIII.	4	2	1	-
XIV.	2	-	1	-
Untypable	4			
Group not established	7 plus 1 +			

NOTE: Antigenically dissimilar strains from different ears of the same dog are indicated thus:- '+' or 'x'

Although the results of this preliminary investigation show (Table 83) that there is a considerable degree of antigenic heterogeneity among Proteus strains, it will be noticed that 32 (48 per cent) of the 67 typable strains fell into a common group, namely Group I. As Krikler (1953) has pointed out, Phase C. strains may react with antigenically dissimilar strains without producing the characteristic line of demarcation at the junction of the spreading halos, an effect which may result in a large number of different strains falling into the same group. It is emphasised, therefore, that in the present investigation all the strains were tested not only against a representative member of each group but against themselves as well.

The significance of these findings is more readily understood when it is remembered that since Weil and Felix (1917, 1918) described in Proteus the presence of somatic and flagellar antigens, Winkle (1945) has distinguished by cross agglutination and cross absorption techniques, 13 'O' antigens and 8 'H' antigens. Perch (1948) examined 540 freshly isolated strains, mainly of the mirabilis variety and identified 49 'O' groups and 19 'H' groups, while Belyavin, Miles and Miles (1951) studied 44 strains, the majority of which were also Pr. mirabilis, and recognised 18 different 'O' antigens and numerous 'H' antigens.

Whilst the frequency of antigenically distinct strains of Pr. mirabilis of canine origin is likewise confirmed, the above results would also indicate that one particular type predominates

predominates in infected ears.

It should also be noted that antigenically identical strains of Pr. mirabilis were isolated from both external ears of no fewer than 17 out of 19 cases of bilateral otitis.

That there is a common source of infection is suggested by the fact that the great majority of bilateral cases of otitis are infected with identical strains, almost 50 per cent of which fall into a common antigenic group. The most probable source of infection in dogs was thought to be the rectum, as it has already been shown in Part II of this work that the coliform organisms in infected ears are predominantly of faecal origin.

Moreover, the presence of Proteus strains in the intestinal contents of man and of animals has been demonstrated by a number of workers of whom Rustigan & Stuart (1945) and Krikler (1953) gave incidence figures for normal human faeces of 26 and 27 per cent respectively. For normal dogs, however, the figure of 37 per cent for Pr. mirabilis (Part I of this work) compares unfavourably with that of Phillips (1955) who reported on 17 (8 per cent) strains from the faeces of 217 dogs with no history of diarrhoea.

In the second part of this investigation the nose, tonsils and rectum were examined in the case of 15 dogs which were suffering from chronic otitis associated with Proteus.

Of these 15 dogs, strains which were antigenically identical to the ear strains were recovered from the nares of 6 dogs, the

the tonsils of 9 dogs and the rectum of 11 dogs. In 6 dogs identical strains were recovered from all four sites.

It has already been shown (Table 60, page 135) that strains of the predominant group, Group I, were present in the ears of 7 dogs, the nares of 3 dogs, the tonsils of 5 dogs and in the rectum of 5 dogs. While these Group I strains were also the commonest type in the rectal swabs of affected dogs, it is interesting to notice that 10 of the 13 rectal strains fell into 4 of the 14 'otitis strain' groups.

Whether Proteus infections in the external ears of dogs arise by cross-infection or by auto-infection from the intestines, these findings point to the latter mechanism. The same opinion is held by Story (1954) with regard to Proteus infections in man.

In the third and final part of this investigation, the 11 strains of Pr. mirabilis from the faeces of normal dogs were considered. Although the number of strains is regrettably small, it is nevertheless of interest to find that 4 strains fell into Group I, one strain into Group III, one strain was in Phase C and the remainder could not be identified with any of the other groups.

The results of this present investigation, taken as a whole, show that:-

(1) There is a considerable degree of heterogeneity among Proteus strains from infected canine ears, as well as from other parts of the body, as no fewer than 14 groups were identified in

in the 67 strains examined.

(2) In spite of this large number of groups, 48 per cent of otitis strains were antigenically identical, all of them falling into Group I.

(3) Proteus strains were present in the faeces of 13 out of 15 chronically infected dogs, 11 of the strains being identical to the ear strains in the same animal.

(4) No fewer than 10 of the 13 rectal strains fell into 4 of the 14 groups for otitis strains. Of these, Group I strains were again the most prevalent.

(5) Although only 5 of the 11 faecal strains from normal dogs could be included in one or other of these 14 groups, 4 strains were shown to be identical to Group I strains.

The above findings tend to confirm the opinion which was expressed earlier in this work that, in many cases of canine otitis, the ear lesions become infected as the result of auto-infection from the intestines.

TABLE 82.

The cultural characters of 100 strains of Proteus.

Character	Pr. mirabilis (90 strains)	Pr. vulgaris (9 strains)	Pr. morganii (1 strain)	TOTAL
	Number of strains giving a positive reaction.			
Urea	90	9	1 L	100
H_2S	90	9	1 L	100
Catalase	90	9	1	100
Nitrates	90	9	1	100
Motility	88	9	1	98
Swarming	88	9	0	97
Gelatin	89	9	0	98
Serum	87	9	0	96
M.R.	90 =	6	1	97
V.P.	44	6	1	51
Citrate	85	4	1	90
Meth. blue	90	9	1	100
Lit. milk :-				
clot	89	7	0	96
digested	89	7	0	96
Indole	0 +	8	1	9
Fermentation of-				
Glucose	90	9	1	100
Maltose	0	9 x	0	9
Sucrose	73	9	0	82
Trehalose	90	9	0	99
Salicin	16	7	0	23
Galactose	89	9	1	99
Glycerol	90	8	1	99
Xylose	89	9	0	98
Laevulose	88	9	1	98 L
Mannose	0	0	1	1

No strain fermented lactose, mannitol, dulcitol, inositol, raffinose, arabinose, rhamnose, dextrin, inulin or sorbitol.

Legend: L = action delayed.
+ = 16 cultures produced indole acetic acid.
x = includes 1 indole negative strain.
= = includes 9 weakly positive strains.

PART VI.b. - SUMMARY:

Of the 182 dog strains 172 (94.5 per cent) were Pr. mirabilis, 9 (5 per cent) were Pr. vulgaris and 1 strain was Pr. morganii. These results are very similar to those of Phillips (1955) who suggested that Pr. mirabilis is the predominant species in dogs, as it is in man. (Rustigan and Stuart, 1945; Damming and Billings, 1942; and Levine, 1942).

The species were adequately determined by their activities in certain sugars, their proteolytic powers and their ability to form indole and hydrogen sulphide. It will be shown later (Part VII) that species identification was also suggested by the strains' in vitro sensitivities to antibiotics.

Unlike Moltke's (1927) human strains, and Phillips' (1955) animals strains, 16 (18 per cent) of the maltose negative dog strains fermented salicin.

Apart from the single morganii strain, other Proteus species were markedly proteolytic, liquefying gelatin media within 72 hours; while vulgaris strains were less active than mirabilis strains in hydrolysing inspissated bovine serum.

Antigenically identical mirabilis strains were distinguished by means of the Dienes phenomenon, and although there was a considerable degree of antigenic heterogeneity, 32 of the 67 strains from infected ears fell into a common group. Most of the strains from the intestines of healthy and affected dogs were antigenically identical to those of this common group. In dogs

dogs with Proteus infected ears identical strains were frequently isolated from the anterior nares, the tonsils and the recta. As the highest incidence of 'pairs', i.e. identical strains from more than one site of the same dog, was given by the rectal swabs, this suggested that infection, by Proteus, of the external ears was probably due to auto-infection from the intestines.

C) COLIFORMS

In this thesis the term "Coliform group" will include members of the genera Escherichia, Klebsiella, Cloaca and Hafnia, as defined by Kauffmann (1954).

It will be recalled that members of these genera were isolated from 6, 12, 14 and 63 per cent respectively of the external and middle ears, the anterior nares and the tonsils of healthy dogs and from 13 per cent of the infected external ears.

TABLE 84

The "Coliform Group" of organisms - and the tissues from which they were recovered

Species	Infected external ears	Outer ears	Middle ears	Nares	Tonsils	Rectum	Total
Escherichia coli	58	3	5	4	15	23	108
Escherichia freundii	1	-	-	-	3	-	4
Klebsiella species	2	3	-	-	2	-	7
Cloaca cloacae	5	-	-	-	1	-	6
Hafnia species	2	1	1	1	-	-	5
(?) Paracolons	-	-	-	-	1	2	3
Total strains per tissue	68	7	6	5	22	25	133

NOTE: Although the term "paracolons" is falling into disrepute, it was necessary to classify three of the strains as such, as they did not readily fall into any of the other four genera.

Definition:

All the organisms included in the "Coliform Group" were motile, or non-motile, Gram negative rods conforming to Kauffmann's (1954) definition of Enterobacteriaeceae. It will not be necessary to redefine each of the four genera, Escherichia, Klebsiella, Cloaca and Hafnia as this has already been done in Part I of this work.

Morphology and Growth Characters:

Neither the shape or size of the bacterial cell nor the appearances of cultures on ordinary solid or liquid media was characteristic of any one species. They were all Gram negative, non sporing rods that grew well on ordinary media and usually fermented glucose promptly with the production of gas. Nitrates were invariably reduced to nitrates. All strains were aerobic, and facultatively anaerobic, with an optimum temperature of 37°C. In broth a uniform turbidity developed within 18 hours and although a slight deposit formed in older cultures, there was no evidence of pellicle formation.

Motility:

Although the presence or absence of motility varies with a number of artificial factors (Wright and Villaneueva, 1953) and is no longer acceptable as an important differential feature of the coli-aerogenes group, the strains were examined for motility so that a comparison could be made with the findings of other

other workers.

Of the 108 E. coli strains 73 (68 per cent) were motile, as were 3 of the 4 freundii strains.

The production of Indole:

There is general agreement that the Indole, M.R., V.P., citrate and Eijkman tests are of value in differentiating the members of the 'Coliform Group'.

Of the 133 strains examined, 110 produced indole, including 107 strains of E. coli and the 3 so-called paracolon organisms, but indole was not produced by E. freundii, Klebsiella, Cloaca or Hafnia species.

M.R. and V.P. reactions:

The M.R. and V.P. tests were carried out on 4 day old glucose phosphate broth cultures, the presence of acetylmethylcarbinol being demonstrated by Barritt's (1936) method. All Escherichia species, and one of the paracolon strains, were M.R. positive whereas only the Klebsiella, Cloaca and Hafnia strains, and 2 of the 3 paracolon organisms, were V.P. positive.

Citrate Utilisation:

Only 9 (8 per cent) of the E. coli strains utilised sodium citrate as a sole source of carbon. With one exception all other members of the "Coliform Group" grew well in Koser's medium after 48 hours incubation at 37°C.

The Eijkman Test:

The ability of coli, but not aerogenes strains, to form gas in glucose broth media was first described by Eijkman in 1904. This, in a modified form, is the basis of an important differential test which is much favoured by some workers and as firmly condemned by others. Although Ware (1951) has shown that the ability to grow at a specified temperature may be a function not only of the strain but also of the constituents of the medium, Topley and Wilson (1955) consider that the 44°C. MacConkey Test, in lactose bile-salt broth, is of greater value than any other single test in picking out typical faecal strains of E. coli.

In this work the test was performed by inoculating a tube of single strength MacConkey broth from an overnight peptone-water culture, the broth being previously heated in a water bath at 37°C. The inoculated tube was immediately placed in a constant temperature water-bath at 44°C and examined after 6 - 24 hours. The presence of gas in the enclosed Durham tube was regarded as a positive reaction and absence of gas, even though growth and acid formation was present, was regarded as a negative reaction. In the absence of gas production after 24 hours, the tubes were reincubated for a further 24 hours. Two control tubes of broth were included, one inoculated with a faecal strain of E. coli and the other with a stock strain of Bact. aerogenes.

Of the 108 E. coli strains, 103 (95 per cent) produced gas in lactose bile-salt medium at 44°C., whereas two cultures, one

one Klebsiella and one atypical Hafnia, gave a trace of gas after 48 hours only. All other strains were negative.

It is now convenient to summarise the results of the Indole, M.R., V.P., citrate and Eijkman reactions as follows:-

TABLE 85

Differentiation of the "Coliform Group" of organisms

Genus	Number of Strains	Gas produced in lactose bile-salt at 44°C	Indole produced	M.R. Positive	V.P. Positive	Growth in Citrate
Escherichia	112	103	107	112	0	13
Klebsiella	7	1+	0	0	7	7
Cloaca	6	0	0	0	6	6
Hafnia	5	1+	0	0	5	4
Paracolons	3	0	3	1	2	3
Total	133	105	110	113	20	33

NOTE: (+) = Weakly and irregularly positive

As 105 (79 per cent) and 110 (83 per cent) of the strains were Eijkman and Indole positive respectively, this suggested that their normal habitat is the intestine. In the "Reports on Public Health and Medical Subjects, No.71" (1956), a number of suggestions are made as to the probable sources of origin of the

the different types of coliform organisms. By adopting these recommendations, it was shown (Table 86) that all but 6 of the 133 strains fell into one of the following ten groups.

TABLE 86

Coliform types and their probable origin

		Eijkman	Indole	M.R.	V.P.	Citrate	Otitis dogs	Normal dogs	Rectum	Probable Source
B. coli	Type I	+	+	+	-	-	54	21	22	Gut
	Type II	-	-	+	-	-	-	-	-	Soil
Intermediate	Type I	-	-	+	-	+	1	3	-	Soil
	Type II	-	+	+	-	+	-	4	1	Soil
B. aerogenes	Type I	-	-	-	+	+	2	7	-	Veg.
	Type II	-	+	-	+	+	-	-	2	Veg.
B. cloacae		-	-	-	+	+	5	1	-	Veg.
Irregular	Type I	-	+	+	-	-	-	1	-	?Gut
	Type II	+	-	+	-	-	1	-	-	?Gut
	Type VI	+	-	-	+	+	2	-	-	?
Other irregular types		+	+	+	-	+	3	2	-	?
		-	-	-	+	-	-	1	-	?
							68	40	25	
Total		105	110	113	20	33		133		

These results show that approximately 81 per cent of the otitic strains and 88 and 55 per cent respectively, of the strains from the recta and other sites in clinically healthy dogs, are of the type that has the intestines as its normal habitat. The significance of this finding was emphasised by comparing the results for the otitic strains with those of a number of other workers, all of whom have stressed the importance of the indole, M.R., V.P., and citrate reactions as a means of identifying the lactose fermenting coli-aerogenes bacilli of faecal origin.

TABLE 87

Showing the percentage of lactose fermenting coliform bacilli from various sources giving the reactions indicated

Author	Source	M.R. (+)	V.P. (+)	Indole (+)	Citrate (+)
≠ Various authors	Faeces, animal and human	90.4	7.87	93.03	6.71
≠ Various authors	Soil	14.7	80.24	33.4	89.6
This work	Canine otitis	96.3	3.3	93.4	9.8

≠ = Quoted by Topley and Wilson (1955)

Lovell (1937, 1955) observed that most strains isolated from calves which had died from "white scour" could be classified biochemically as Bact. coli type 1. He considered this to be the main faecal type as Wilson et al (1935) found that of 125 strains of coli-aerogenes isolated from cow dung, 104 proved to be Bact. coli type 1.

In view of these findings and from the results in Tables 86 and 87, it would appear that most of the coliform organisms from infected canine ears were the faecal type 1 of E. coli. This suggested that infection of the primary ear lesion was probably due to contamination from an external source or by auto-infection from the intestines.

Fermentation of sugars:

The fermentative activities of the species within the various genera can be described in the following general terms.

E. coli:

All but 2 strains, one of which failed to act on maltose and the other on arabinose, produced acid and gas in glucose, lactose, maltose, mannitol, sorbitol, trehalose, rhamnose, glycerol, laevulose, mannose and galactose. Two strains had no effect on xylose while the reactions were variable in raffinose, salicin, dulcitol and sucrose. None of the strains fermented inulin and only 1 strain fermented inositol.

E. freundii:/

E. freundii:

Most E. freundii strains fermented all of the sugars except salicin, inulin and inositol.

Klebsiella:

None of the Klebsiella strains attacked dulcitol and inulin although all of them, with one exception, fermented the other sugars, including inositol.

Cloaca:

These organisms were very similar to the Klebsiella strains although they did not ferment either dulcitol or inositol. One of the strains failed to produce acid in lactose.

Hafnia:

All of the 5 Hafnia strains failed to act on lactose, dulcitol, inositol and inulin. The reactions in sucrose and salicin were variable, but all other sugars were promptly fermented with the production of both acid and gas.

Paracolons:

This term is used to describe three strains which could not be placed with members of the other four genera as they failed to ferment lactose but grew well in Koser's medium and produced indole. They also failed to ferment dulcitol, inositol, salicin, arabinose, rhamnose and xylose, whereas saccharose, raffinose and sorbitol were irregularly attacked.

The various fermentative types are shown in Table 88.

TABLE 88.

Biochemical types within the coliform group.

	Escherichia coli								Escherichia freundii		Klebsielle	Cloaca		Hafnia		Paracolons.
	Type								Type		Type	Type		Type		Type
	1.	2.	3.	4.	5.	6.	7.	8.	1.	2.	1.	1.	2.	1.	2.	1.
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Maltose	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-
Inositol	∓	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Sucrose	+	-	-	+	+	-	-	+	+	v.	+	+	+	v.	v.	v.
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	v.	v.	v.
Salicin	+	+	-	-	+	+	-	-	v.	-	+	+	+	+	-	-
Raffinose	v.	v.	v.	v.	+	-	-	+	+	+	v.	v.	+	v.	v.	v.
Arabinose	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	-
Xylose	±	+	+	+	+	+	v.	+	+	+	+	+	+	+	+	-
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	+	+	+	v.	+	+	+	+	-	-	-	-	-	-	-	+
Otitis	25	17	5	5	1	3	1	1	1	-	2	5	-	1	1	-
Normals	11	5	3	5	2	1	-	-	1	2	5	-	1	1	2	1
Rectum	6	7	-	4	2	2	1	1	-	-	-	-	-	-	-	2
Total	42	29	8	14	5	6	2	2	2	2	7	5	1	2	3	3

In addition, all strains fermented trehalose, glycerol, galactose, laevulose and mannose.

Legend: (±) = Positive reaction but with an occasional negative strain.
(∓) = Negative reaction but with an occasional positive strain.
(v.) = Reaction variable.

Otitis = Includes only strains from infected ears.
Normals = Includes strains from the ears, nose and tonsils of healthy dogs.
Rectum = Includes selected strains from the recta of healthy dogs.

Another method of grouping the coliforms is by the ordinary fermentation tests in sugars and, while classification by this method has never assumed any practical importance, it is nevertheless interesting to compare the dog strains with the named species of other workers.

On the basis of their sugar reactions in salicin and saccharose, Bact. coli may be divided into a number of varieties of which the following four types are probably the most common:- var commune, var communius, var neapolitanum and var acidi-lacti.

TABLE 89

The classification of the coliforms by their action on saccharose and salicin

Species (Varieties)	Acid and Gas Produced in:- Sucrose	Salicin	Source and number of strains giving the reaction indicated: Infected ears	Healthy dogs: Outer ears	Middle ears	Nose	Tonsils	Rectum	Total
Bact. coli									
var neapolitanum	+	+	23	2	3	1	7	8	44
var coli communius	+	-	5	0	2	1	2	5	15
var coli commune	-	+	25	1	0	2	3	8	39
var acidi-lacti	-	-	5	0	0	0	3	2	10
			58	3	5	4	15	23	108

Haemolysis:

It is well known that some strains of E. coli are actively haemolytic. Kauffmann (1954) has shown that many strains of pathological origin are not only haemolytic but are toxic to mice and capable of causing necrosis when injected into the skin of a rabbit. He also noted that these haemolytic strains were particularly common in "O" groups, 2, 4 and 6, of which group 6 contains the most toxic types. It is believed that the haemolytic activity is due, not to a filterable haemolysin, but to the growth of the organism.

The haemolytic effects of a representative number of strains of the "Coliform Group" from infected ears were investigated by Kauffmann's (1954) methods, using horse erythrocytes as the indicator.

Fifty strains were studied of which 40 were E. coli, 4 were E. freundii and 6 were C. cloacae.

The results obtained showed that all the freundii and cloacae strains were non haemolytic and that 23 (57.5 per cent) E. coli strains haemolysed horse red cells within 24 hours.

Hydrolysis of urea:

Seven of the 108 E. coli cultures showed evidence of urea decomposition although in every case the reaction was faint and delayed. Moreover, 1 out of 4 freundii, 5 out of 7 Klebsiella, 4 out of 6 cloacae and 1 out of 3 'paracolon' strains hydrolysed urea. All 5 Hafnia strains were negative.

Liquefaction of gelatin:

Although none of the E. coli strains liquefied gelatin within 28 days and are described as being negative, 8 strains liquefied the medium some four to six weeks later.

Apart from the 6 cloacae strains, and one of the 'paracolon' organisms, all of the other species were also negative.

Hydrogen sulphide production:

The only species to produce appreciable amounts of H_2S were the 4 strains of Klebsiella and one of the 'paracolons'. Except for three Klebsiella strains which produced traces of hydrogen sulphide, all other members of the "Coliform Group" were negative.

Catalase Production and the Reduction of Nitrates:

All strains produced catalase and reduced nitrates to nitrites within 18 hours.

Milk Media:

All strains reduced methylene blue milk and produced small amounts of acid in litmus milk. Only 8 strains failed to produce a coagulum in the latter medium, the production of which was weak and delayed in the case of the E. coli strains. Litmus milk was reduced within 24 hours by only 20 (18.5 per cent) of the E. coli and 4 of the 6 cloacae strains.

Details of the results of the different tests to which members of the "Coliform Group" were submitted are summarised, for convenience, in Table 90.

TABLE 90.

The characters of the "Coliform Group" of organisms.

Character	Escherichia		Klebsiella	Cloaca	Hafnia	Paracolons	Total
	coli (108)	freundii (4)	(7)	(6)	(5)	(3)	(133)
Motility	73	3	0	6	2	1	85
Indole	107	0	0	0	0	3	110
M.R.	108	4	0	0	0	1	113
V.P.	0	0	7	6	5	2	20
Citrate	9	4	7	6	4	3	33
Eijkman	103	0	1+	0	1+	0	105
Methylene blue	108	4	7	6	5	3	133
Litmus milk -							
Acid	108	4	7	6	5	3	133
Clot	106	4	5	6	1	2	124
Reduced	20	0	0	4	0	0	24
Urea	7	1	5	4	0	1	18
Gelatin	0	0	0	6	0	1	7
Hydrogen sulphide	0	4	3	0	0	1	8
Catalase	108	4	7	6	5	3	133
Nitrates	108	4	7	6	5	3	133
Haemolysis	23 x	0	-	0	-	-	
Fermentation of -							
Glucose	108	4	7	6	5	3	133
Lactose	108	4	7	5	0	0	124
Maltose	107	4	7	6	5	3	132
Mannitol	108	4	7	6	5	3	133
Dulcitol	92	2	0	0	0	0	94
Inositol	1	0	7	0	0	0	8
Saccharose	60	3	7	6	2	1	79
Sorbitol	108	4	7	6	3	2	130
Salicin	82	1	7	6	2	0	98
Raffinose	53	4	6	5	2	1	71
Arabinose	107	4	7	6	5	1	130
Trehalose	108	4	7	6	5	3	133
Xylose	106	4	7	6	5	1	129
Inulin	0	0	0	0	0	0	0
Rhamonse	108	4	7	6	5	1	131
Glycerol	108	4	7	6	5	3	133
Galactose	108	4	7	6	5	3	133
Laevulose	108	4	7	6	5	3	133
Mannose	108	4	7	6	5	3	133

(+) = weak and irregularly positive.
(x) = only 40 E. coli strains studied.

Note: The above results show the number of strains giving a positive reaction. The numerals in brackets indicate the number of strains examined.

PART VI.c. - SUMMARY:

The "Coliform Group" of organisms from normal and infected dogs have been discussed under the genera Escherichia, Klebsiella, Cloaca and Hafnia. Although there was no evidence of Arizona species, from any of the tissues examined, three non-lactose fermenting strains which could not be included in the above classification were described as 'paracolon organisms".

Escherichia:

The members of this genus consisted of 108 strains of E. coli and only 4 of E. freundii.

The E. coli strains were very similar in character to those of Kauffmann (1954) in that they fermented lactose, glucose and mannitol but rarely inositol. Acid production was variable in dulcitol (85 per cent), saccharose (55.5 per cent), salicin (76 per cent) and raffinose (49 per cent). They all reduced nitrates to nitrites but were negative to the V.P., H_2S., gelatin and urea tests. Indole was formed in peptone water by 99 per cent of strains, while 92 per cent failed to utilise citrate as a sole source of carbon.

The freundii strains were distinguished from E. coli by the fact that they did not produce indole in peptone water or gas in lactose bile medium at 44°C. but were able to grow well in Koser's citrate medium after 18 hours at 37°C. Their fermentative activities were, however, very similar to those of E. coli.

Klebsiella:

Seven Klebsiella strains were isolated, all of which were typical of the genus in that they were V.P. and citrate positive, non-motile rods which did not produce indole in peptone water. With one exception they fermented all of the sugars except inulin and dulcitol. There was no liquefaction of gelatin but 5 strains hydrolysed urea and 3 strains produced appreciable amounts of hydrogen sulphide.

Cloaca:

All of the 6 cloacae strains liquefied gelatin slowly, produced acetylmethylcarbinol and utilised citrates as a sole source of carbon. They produced neither indole nor hydrogen sulphide and 4 strains decomposed urea. Although none of the strains fermented dulcitol or inositol, it is interesting to observe that 5 of the 6 strains fermented lactose.

Hafnia:

The members of the genus Hafnia resembled the Cloaca species in that they were motile rods which produced acetylmethylcarbinol and were usually able to grow in Koser's medium. Although they also failed to form indole they differed from the Cloaca strains by failing to hydrolyse urea or gelatin.

Paracolon organisms:

Although the three so-called paracolon strains produced indole, they resembled Hafnia species by failing to act on lactose,

lactose, dulcitol or inositol.

Apart from E. coli, members of the genera Escherichia, Klebsiella, Hafnia and Cloaca were thought to be of little importance in canine otitis. However, as 85 per cent of the coliform organisms from infected ears, compared with only 6 per cent from clinically healthy ears, were E. coli of which at least 81 per cent were probably of faecal origin, this suggested that their presence in otitic material was the result of auto-infection from the intestines.

d) STAPHYLOCOCCI

Although they are frequently associated with localised lesions such as abscesses, carbuncles, infected interdigital cysts and otitis, it is often very difficult to assess the specific role of the staphylococci present. This may be due to the fact that, whereas staphylococci are not so frequent in animal as in human infections (Hagen and Bruner, 1951), the organisms when present are often associated with other bacteria. This is especially true of a number of suppurative conditions in animals.

Another factor which adds to the difficulty of interpretation is that the pathogenic animal staphylococci have not received the same amount of detailed study as have the pathogenic human strains. As a result, the importance of a particular staphylococcus in an animal lesion may be overlooked merely because it does not conform to the pattern of the typical pathogenic Staphylococcus aureus (Staph. pyogenes var aureus) of man.

Both Hagen (1951) and Richou et al (1956) suggest that there is no reason to doubt that human and animal pathogenic staphylococci are identical. In recent months Rountree, Freeman and Johnson (1956) studied the nasal carriage of Staphylococcus aureus by various domestic and laboratory animals and found that all phage typable strains, from animal nasal carriers, were similar to human strains. This, they thought, suggested that the presence of staphylococci in the nares of animals was due to contact with man.

On the other hand (Foggie, 1947) reported that his strains from cases of pyaemia in lambs were apparently characteristic of "sheep staphylococci" as opposed to Staph. aureus from other animals.

Minett's (1936) observations that coagulase positive dog staphylococci showed marked proteolytic activity and produced little alpha toxin, if any, suggested important differences between dog and human pathogens. Minett's findings were largely confirmed by Smith, 1947, who also reported that his dog strains rarely produced pigment.

The ability of some strains to produce pigment was used as a basis of classification by Rosenbach (1884) and Passet (1885) who described aureus, albus and citreus strains. While it is accepted that the great majority of human and most animal pathogenic staphylococci are able to produce the golden or aureus type of pigment, albus variants are not uncommon.

Other characteristics which have been used by various workers as a means of classifying staphylococci include their activities in milk media, their proteolytic powers and their ability to ferment a number of 'sugars' with the production of acid but not gas.

Andrews and Gordon (1905 - 06) stressed the fermentation of maltose, lactose, glycerol, and mannitol but Winslow, Rothberg and Parsons (1920) thought that the production of acid from lactose was of most value.

Topley and Wilson (1946) and Mackie and McCartney (1949) agree that most Staph. aureus strains of human origin ferment both lactose and mannitol and, although this is also true of most animal strains (Smith, 1947) and of mannitol with bovine strains (Edwards and Rippon, 1957), it will be shown that many dog strains failed to ferment mannitol or did so only after three days' incubation.

The coagulase activity of staphylococci which was first reported by Loeb (1903 - 04) has received the attention of numerous workers, notably Chapman, Berens, Peters and Curcio (1934), Cruickshank (1937) and Fisk (1940), and it is agreed that there is a close correlation between coagulase production and pathogenicity, although as Edwards and Rippon (1957) have pointed out, no standard for the application of the test or even of the plasma to be used has been laid down.

Notwithstanding the value of the coagulase test, staphylococci may produce a number of other active agents, the formation of which is mainly confined to pathogenic strains. These factors include leucocidin, enterotoxin, fibrinolysin, hyaluronidase and at least three haemolytic toxins.

The production of alpha toxin has been studied in some detail and while Bryce and Rountree (1936) draw attention to the close relationship between this and pathogenicity in human strains, Marks (1952) goes a step further and claims that the ability of a strain to form alpha toxin is a better indication of pathogen-

pathogenicity than is the production of coagulase.

The beta toxin which is characteristic of animal strains (Minett, 1936) differs from alpha toxin (Flaum and Forssman, 1936) and is quite unrelated to pathogenicity (Christie and North, 1941).

A third toxin, the gamma toxin of Smith and Price (1938), resembles the alpha-two toxin of Morgan and Graydon (1936) and is thought to be identical to the delta haemolysin, of Williams and Harper (1947). The presence of this haemolysin is of significance as it is frequently found in cultures of staphylococci that produce alpha or beta toxins but never in cultures that produce neither of these, or by strains that are coagulase negative.

Several of these factors, and the effects they produce, will be referred to in the discussion of the various tests to which dog and other animal strains of staphylococci were submitted.

Four hundred strains of aerobic, catalase positive, Gram-positive cocci which grew well and produced large opaque colonies were studied during this investigation. The source of these strains is as follows:-

Infected canine ears	200 strains
Healthy canine ears	84 strains
Anterior nares of healthy dog	17 strains
Tonsils of healthy dogs	11 strains
Other lesions in the dog	23 strains
Bovine lesions	50 strains
Lesions in other animals	15 strains
Total	400 strains

Morphology:

All strains were Gram positive in young cultures although many became Gram negative as the cultures aged. The diameter of the individual coccus varied from approximately 0.7 - 1.5 μ.

Temperature range:

All grew well at 25°C. and at 37°C., although the majority grew best at 37°C.

Type of growth:

On nutrient agar the colonies were round, low, convex and opaque, usually with a smooth surface and entire edge. In nutrient broth most strains showed a uniform general turbidity with a variable amount of deposit. After 24 hours at 37°C. a ring pellicle was formed on the surface of the culture but a few strains produced only a very slight degree of turbidity with a moderately heavy granular deposit.

The production of catalase:

Each strain was tested for its ability to produce catalase before it was finally classified as a staphylococcus. The reliability of this test as a means of differentiating the Gram-positive cocci (Coccaceae) was demonstrated by Isaacs and Scouller (1948) who found all (146) of their strains, classified as micrococci, staphylococci and Sarcina, to be catalase positive. Streptococci, on the other hand, were consistently negative.

Catalase activity was also considered by Shaw, Stitt and Cowan (1951) to be an important aid in the classification of staphylococci. All the strains from infected canine ears were catalase positive.

Fermentation reactions:

Although the fermentation of sugars, other than lactose, maltose and mannitol by staphylococci is probably unrelated to pathogenicity, it has yet to be shown that this applies equally to dog strains.

TABLE 91

The fermentation reactions of staphylococci from infected ears

	Coagulase Positive (50 strains)		Coagulase Negative (10 strains)	
	Acid only (+)	No Acid (-)	Acid only (+)	No Acid (-)
Glucose	50	0	10	0
Lactose	50	0	6	4
Maltose	46	4	5	5
Mannitol	32	18	8	2
Dulcitol	0	50	0	10
Inositol	0	50	0	10
Sucrose	50	0	10	0
Sorbitol	14	36	5	5
Salicin	0	50	0	10
Raffinose	0	50	0	10
Arabinose	0	50	0	10
Trehalose	48	2	6	4
Rhamnose	0	50	0	10
Glycerol	49	1	7	3
Inulin	0	50	0	10
Galactose	50	0	10	0

The fact that acid was produced in lactose, maltose and mannitol by 100 per cent, 92 per cent and 64 per cent of strains respectively appeared to confirm an earlier impression that many coagulase positive canine staphylococci did not ferment mannitol. In addition, the mannitol fermenters usually took 3 days to show visible acid change.

As there did not appear to be a positive correlation between coagulase activity and sugar fermentation, it was decided to discontinue all fermentation tests except those with lactose, maltose and mannitol, as a method of classification.

TABLE 92

The fermentation of lactose, maltose and mannitol by animal staphylococci

Coagulase Positive Strains

Origin of strains	Number of Strains Examined	Lactose +	Lactose −	Maltose +	Maltose −	Mannitol +	Mannitol −
Dogs - infected ears	156	152	4	142	14	97	59
Healthy dogs - ears	36	36	0	33	3	13	23
nose	12	11	1	12	0	9	3
tonsils	4	4	0	4	0	2	2
Dogs - other lesions	19	19	0	16	3	11	8
Bovine lesions	42	42	0	42	0	41	1
Other animal lesions	13	13	0	11	2	12	1
Total	282	277	5	260	22	185	57

	Number of Coagulase +ve Strains	Acid in Lactose	Acid in Maltose	Acid in Mannitol
All dog strains	227	222(98%)	207(91%)	132(58%)
All animal strains	55	55(100%)	53(96%)	93(96%)

TABLE 93

The fermentation of lactose, maltose and mannitol by animal staphylococci

Coagulase Negative Strains

Origin of strains	Number of Strains Examined	Lactose +	Lactose -	Maltose +	Maltose -	Mannitol +	Mannitol -
Dogs - infected ears	44	33	11	27	17	24	20
Healthy dogs - ears	48	28	20	34	14	13	35
nose	5	2	3	4	1	2	3
tonsils	7	2	5	5	2	1	6
Dogs - other lesions	4	3	1	2	2	1	3
Bovine lesions	8	6	2	5	3	3	5
Other animal lesions	2	2	0	2	0	1	1
Total	118	76	42	79	39	45	73

	Number of Coagulase -ve Strains	Acid in Lactose	Acid in Maltose	Acid in Mannitol
All dog strains	108	68(63%)	72(67%)	41(38%)
All animal strains	10	8(80%)	7(70%)	4(40%)

These results show coagulase positive strains to be more active in liquid carbohydrate media than coagulase negative strains. They also show that most animal pathogenic staphylococci (but not dog strains) ferment lactose, maltose and mannitol, which suggests that in this respect at least they resemble human strains rather

rather than dog strains.

The coagulase positive dog staphylococci whilst usually fermenting lactose and maltose (98 and 91 per cent respectively) frequently fail to produce acid from mannitol. The figure of 58 per cent shows that there is little correlation between the coagulase activity of a staphylococcus of canine origin and its ability to ferment mannitol. Moreover, many of the mannitol fermenting dog strains failed to show the presence of acid until after three days' incubation, whereas bovine and other animal staphylococci which fermented this 'sugar' usually did so within twenty-four hours.

Liquefaction of gelatin:

Gelatin was stab inoculated from 18-hour nutrient agar cultures. The inoculated medium was incubated in the dark at room temperature for a period of four weeks, unless liquefaction occurred before this.

The fact that staphylococci of canine origin are markedly proteolytic was first described by Minett (1936) who also observed that other animal staphylococci may show proteolytic powers to a lesser degree. However, as Smith (1947) points out, the ability of animal strains to liquefy gelatin does not, by itself, differentiate the pathogens from the non-pathogens. Of his 110 pathogenic staphylococci from animals 106 strains liquefied gelatin, as did 35 coagulase negative strains. On the other hand, Edwards and Rippon (1957) who classified 381 bovine mastitis

mastitis strains by phage typing and coagulase activity found that only 15 strains liquefied gelatin within 5 days at 37°C. Although the coagulase positive staphylococci examined by Shaw et al (1951) were obtained from a variety of sources which included humans, animals, air, ice-cream, milk and other foodstuffs, all 143 strains liquefied gelatin. The same was true of all their V.P. negative, coagulase negative strains.

Such results suggest that the ability of a staphylococcus to liquefy gelatin is no longer a reliable indication of its pathogenicity.

TABLE 94

Liquefaction of gelatin by animal staphylococci

	Coagulase Positive			Coagulase Negative		
Source of Strains	Number Examined	Gelatin Liquefied +	Gelatin Liquefied −	Number Examined	Gelatin Liquefied +	Gelatin Liquefied −
Dogs - infected ears	156	154	2	44	21	23
Healthy dogs -						
ears	36	36	0	48	25	23
nares	12	12	0	5	3	2
tonsils	4	4	0	7	3	4
Dogs - other lesions	19	19	0	4	4	0
Bovine lesions	42	42	0	8	8	0
Other animal lesions	13	12	1	2	2	0
Total	282	279	3	118	66	52
		(99%)	(1%)		56%	44%

With only 3 exceptions, all coagulase positive animal staphylococci liquefied gelatin within 28 days, whereas only 56 per cent of the coagulase negative strains did so. None of the 10 coagulase negative bovine and other animal strains failed to liquefy gelatin while 44 per cent of the non-pathogenic dog strains showed no proteolytic activity in the same medium.

Minett's (1936) findings with regard to dog strains were confirmed, the great majority of cultures showing evidence of surface liquefaction within 24 hours.

Hydrolysis of solid serum:

The marked proteolytic power of dog staphylococci was confirmed by the fact that more than 90 per cent of the coagulase positive strains liquefied bovine serum slopes within 48 hours.

TABLE 95

The hydrolysis of solid serum by animal staphylococci

	Coagulase Positive			Coagulase Negative		
	Number of Strains	Serum Liquefied +	Serum Liquefied -	Number of Strains	Serum Liquefied +	Serum Liquefied -
Dogs - infected ears	156	146	10	44	20	24
Healthy dogs - ears	36	36	0	48	13	35
nares	12	12	0	5	0	5
tonsils	4	4	0	7	3	4
Dogs - other lesions	19	17	2	4	4	0
Bovine lesions	42	29	13	8	5	3
Other animal lesions	13	10	3	2	2	0
Total	282	254 (90%)	28	118	47 (40%)	71

	Coagulase Positive		Coagulase Negative	
	Number of Strains	Serum Liquefied	Number of Strains	Serum Liquefied
All dog strains	257	215(84%)	108	40(37%)
All animal strains	55	39(71%)	10	7(70%)

Most of the dog staphylococci that liquefied solid serum did so actively and within 24 hours, liquefaction of the surface of the serum slope, however slight, being taken as evidence of the strain's proteolytic activity. The majority of the bovine

bovine staphylococci liquefied the medium weakly, if at all, and when they did so the effect was rarely seen before four days' incubation at 37°; nor did prolonged incubation increase the effect.

Therefore, the difference of 14 per cent between the number of dog and other animal strains that liquefy serum is misleading when one considers the marked activity of the canine staphylococci compared to the correspondingly weak effect of the bovine and other animal strains.

The coagulase test:

The ability of certain staphylococci to produce coagulase is generally regarded as a reliable indication of their pathogenicity. (Chapman, et al, 1934; Cruickshank, 1937; Fairbrother, 1940; Christie et al, 1940; and Field & Smith, 1945). Whilst not minimising the importance of other factors, e.g. alpha toxin, the initial infectivity of staphylococci largely depends on their power to produce coagulase, so that without this power the virulence factors are probably of little avail (Smith, Hale & Smith, 1947). In addition, the presence or absence of coagulase affords a convenient method of classification.

All staphylococci in this survey were tested for their ability to produce coagulase by adding to 0.5 ml. of a tenfold dilution of rabbit plasma in saline, 5 drops of an overnight nutrient broth culture. The tubes were incubated in a water-bath at 37°C. and were examined at half an hour, one hour, three hours

hours and six hours, and finally after standing on the bench at room temperature overnight. Appropriate controls were set up with each test. This method is very similar to that of Fisk (1940).

TABLE 96

The coagulase activity of animal staphylococci

Source of Strains	Number of Strains	Coagulase Positive		Coagulase Negative	
		No.	%.	No.	%.
Dogs - infected ears	200	156	(78)	44	(22)
Healthy dogs -					
ears	84	36	(43)	48	(57)
nares	17	12	(71)	5	(29)
tonsils	11	4	(36)	7	(64)
Dogs - other lesions	23	19	(83)	4	(17)
Bovine lesions	50	42	(84)	8	(16)
Other animal lesions	15	13	(87)	2	(15)
Total:	400	282	(71 %)	118	(30%)

The significance of the staphylococci in infected dogs' ears is shown by the fact that 78 per cent of strains coagulated rabbit plasma. This figure compares favourably with that of 83 per cent of coagulase positive strains from other lesions in dogs. It can be said, therefore, that almost 80 per cent of the

the staphylococci present in cases of otitis in the dog are potentially pathogenic. The reliability of rabbit plasma as an indication of coagulase activity is confirmed by the fact that of the staphylococci recovered from lesions of animals, other than dogs, a similar number (85 per cent) produced coagulase. (Table 96).

It is also interesting to notice that 71 per cent of the staphylococci in the anterior nares of healthy dogs were coagulase positive but that more than half (57 per cent) of the strains from healthy ears and 64 per cent from the tonsils failed to show evidence of coagulase. That the majority of nasal carrier staphylococci are coagulase positive may be due to the fact that the "healthy dogs" were hospitalised patients in the Surgical Block of this School. The significance of this observation and that of Rountree et al (1956) has already been discussed in Part II.

All the above results depend on the activity of staphylococcal cultures in rabbit plasma. Doubts arose as to the suitability of this plasma when it was realised that only 14 of 156 coagulase positive dog strains produced the aureus type of pigment on milk agar medium which characterises human and most animal pathogenic staphylococci. However, Smith (1947) and Richou, Guilhon and Bousicaux (1956) have reported that many of their pathogenic dog strains failed to produce pigment and it is also known that albus variants of human Staph. aureus may occur.

The fact that only 34 per cent of our coagulase positive dog strains produced toxins resembling alpha toxin suggested that there was no correlation between coagulase activity and the presence of alpha toxin, although numerous workers have emphasised the correlation between alpha toxin production and pathogenicity. Cowan (1938) reported that all his coagulase positive human strains produced alpha toxin, while Gillespie, Devenish and Cowan (1939) found that all coagulase positive staphylococci from the nose and skin of humans produced alpha toxin but that none of 198 coagulase negative strains did so.

Similar findings have been reported by Schwabacher, Cunliffe and Williams (1945), Christie, North and Parkin (1946), Williams and Harper (1946) and Christie and Keogh (1940). These workers found alpha toxin to be produced by 91 per cent, 100 per cent, 93 per cent and 90 per cent respectively of human pathogenic staphylococci.

In addition, Cowan (1938) reported that 9 out of 13 animal strains produced alpha toxin; Smith (1947) that 110 animal pathogens produced alpha toxin alone or in combination with beta toxin and Richou et al (1956) that 30 of 41 canine pathogens were either alpha or alpha and beta toxin-producers. Other workers, including Foggie (1947), Jones (1955) and Edwards et al (1957) have found that most pathogenic animal staphylococci produce alpha toxin alone or in combination with other toxins.

Because of the overwhelming evidence that coagulase positive human and animal staphylococci usually produce alpha toxin,

toxin, whereas the majority of our coagulase positive dog strains did not, it was thought necessary to re-examine both effects in some detail.

The coagulase test:

A small number of pathogenic staphylococci from different animals were tested in different dilutions of plasmas from various sources. The blood samples were obtained from a horse, cow, goat, sheep, guinea-pig, three human volunteers and two dogs. As a result of this preliminary investigation, it was decided to carry out the test proper using 1/10 dilutions of human, horse, sheep, dog and rabbit plasma.

The 100 strains used were identified as follows:-

75 dog pathogens of which -

1	produced	alpha toxin only
1	"	beta " "
3	"	alpha and beta toxins only
5	"	alpha and delta " "
20	"	alpha, beta and delta toxins
41	"	beta and delta toxins only
4	"	beta toxin and a trace only of delta toxin

25 pathogens from other animals

Hen	2	alpha and delta strains
Horse	2	alpha, beta and delta strains
Sheep	3	" " " "
Mink	1	" " " "
Bovine	10	" " " "
Bovine	2	alpha and delta strains
Bovine	5	beta and delta strains

In each test 10 coagulase negative cultures were used as negative controls.

TABLE 97

The coagulation of plasma by animal staphylococci

Dog Pathogens (75 strains)

Source of Plasma	TOTAL coagulase positive.		COAGULATION TIME IN HOURS							
			1		3		6		18	
	Number of Strains	%	Number of Strains	%	Number of Strains	%	Number of Strains	%	Number of Strains	%
Rabbit	75	100	7	9	41	55	16	21	11	15
Human	10	13	6	8	2	3	-	-	2	3
Horse	64	85	4	5	39	52	12	16	9	12
Dog	72	96	27	36	33	44	6	8	6	8
Sheep	70	93	23	31	32	43	3	4	12	16

Other animal pathogens (25 strains)

Rabbit	25	100	24	96	-	-	1	4	-	-
Human	23	92	23	92	-	-	-	-	-	-
Horse	25	100	3	12	17	68	4	16	1	4
Dog	22	88	19	76	2	8	1	4	-	-
Sheep	23	92	18	72	-	-	-	-	5	20

Whilst these results confirmed that most dog pathogenic staphylococci coagulate rabbit, dog, sheep and horse plasmas, in that order, the fact that other animal pathogenic staphylococci coagulated rabbit and horse, sheep and human, and dog plasmas, in that order, confirmed the suitability of rabbit plasma for demonstrating coagulase production by all animal staphylococci.

They also showed that dog strains differ from other animal strains in that they rarely coagulate human plasma. Similar results were obtained with human plasma and with crude solutions of human fibrinogen when canine strains were tested by Berger's (1943) slide techniques. It is possible that the coagulability of human plasma by human and other animal pathogenic staphylococci is in some way connected, not only with the presence of coagulase but by the influence of alpha toxin which is usually produced by these strains but not by dog strains.

It was also noticed that, with one exception, all "other animal" pathogenic staphylococci coagulated rabbit plasma within half an hour, whereas only five dog strains did so. The majority (55 per cent) of dog strains required from one to three hours' incubation, although eleven strains required eighteen hours, five of which produced beta toxin only, and beta toxin plus minimal amounts of delta haemolysin. This suggests that the coagulase of alpha-producing staphylococci is more active and is produced in greater amounts than in dog or other strains which do not produce this toxin.

Only ten dog strains coagulated human plasma, six of them within an hour, another two within three hours and the r emainder after 18 hours. Of the 8 strains which acted within 3 hours, one was the alpha toxin strain, five produced alpha toxins and delta toxins and two alpha and beta toxins. Of the remaining 65 strains which were mainly of the beta delta type or the so-called "dog alpha beta delta type" (vide infra), only two strains caused coagulation of human plasma, and then only after overnight incubation at room temperature.

The remainder of the dog strains failed to show definite evidence of coagulation of human plasma, although in a few cases a number of large flakes settled at the foot of the tubes, without other evidence of a coagulum being formed. Horse plasma was less reliable than rabbit plasma for identifying pathogenic dog staphylococci, as only 64 or 85 per cent of strains were positive. Moreover, the coagulum took longer to form than did the rabbit plasma.

Dog plasma was reliable when used with strains of canine origin. Coagulation occurred within the hour in 37 per cent of cases and was, therefore, more rapid in its action than rabbit and horse plasma, but three dog and three other-animal strains which were thought to be pathogenic failed to clot dog plasma.

Sheep was preferred to cattle or goat plasma, although the results obtained in the preliminary tests were very similar, whichever plasma was used.

Both sheep and dog plasmas gave approximately the same number of coagulase positive dog strains, although more strains of other animal origin were coagulated by the latter plasma (72 and 84 per cent respectively, within 3 hours). Moreover, a large number of "other animal" strains failed to coagulate sheep plasma within six hours, whereas with dog plasma all the strains showed coagulase to be produced without the need for overnight incubation at room temperature.

Staphylococcal toxins:

Certain staphylococci when grown under suitable conditions produce a filterable toxin which gives rise to a series of effects, many of which provide an indication of the strain's pathogenicity. That these various effects are due to one and the same toxin has been postulated by Burnet (1929) and others, but in recent years opinion has inclined to the opposite view.

Parker (1924) reinvestigated the whole problem of staphylococcal toxins and, as a result, it is accepted that a staphylococcal toxic filtrate is haemolytic, has a destructive action on leucocytes, causes skin necrosis in rabbits and guinea pigs (when injected intradermally) and, when injected intravenously into rabbits and mice, causes an acute and fatal toxaemia. The toxin production of human pathogenic staphylococci has received intensive study in recent years and a number of reports include the behaviour of staphylococci of animal origin.

As dog strains appeared to be weak coagulase producers compared with the staphylococci from other animal sources, an attempt was made to identify some of the toxins in the hope that the potential pathogenicity of the canine strains might be confirmed irrespective of the amount of coagulase produced.

This approach is justified in view of the observations of Chapman, Berens, Peters and Curcio (1934) that there is a close relationship between alpha toxin production and pathogenicity. Of a similar opinion are Cowan (1938; Gillespie (1939); Bryce and Rountree (1936); McFarlane (1938); Christie and Keogh (1940) and Gillespie & Simpson (1948).

Of more interest to this present work is Christie's (1946) report that 93 coagulase positive animal staphylococci all produced alpha toxin. This was confirmed by Edwards et al (1957) who noted that of 381 coagulase positive bovine mastitis strains, 335 produced alpha, beta and delta haemolysins and that the remaining 46 strains formed alpha and beta haemolysins.

Bryce and Rountree found that animal strains produced beta, as well as alpha toxin, as did Cowan (1936) who found that of 13 coagulase positive animal strains, two produced only alpha toxin, four only beta toxin and seven both alpha and beta toxins.

Williams and Harper (1946) noticed that only 2 per cent of human pathogenic staphylococci produced beta toxin and a year later that only 42 of 2,267 known human pathogenic strains produced beta toxin. This later report emphasised the importance of

of Minett's (1936) observation that beta toxin production was characteristic of animal staphylococci. Minett studied a few dog strains and found that toxins prepared from some of them gave anomalous results. He also remarked that animal strains in general produced not only both alpha and beta toxins but as much alpha toxin as did human strains, but that dog strains were exceptional in this respect. Minett concluded that while his dog strains produced beta toxin and only very small or undetectable amounts of alpha toxin, there was also evidence that certain dog strains produced a third unidentified toxin.

In 1951, Marks made special reference to anomalous haemolysins including the delta haemolysin of Williams and Harper (1947). During this study he examined Minett's dog D4 strain and was of the opinion that it produced both beta toxin and delta haemolysin and that the unidentified toxin was identical to Williams and Harper's delta toxin. In summarising his findings Marks suggested that Minett's toxin, the alpha-two toxin of Morgan and Graydon (1936) and the delta haemolysin are identical.

The gamma toxin which was first described by Smith and Price (1938) is different from the alpha and beta toxins but is, according to Van Heyningen (1950), probably the same as the alpha-two toxin of Morgan and Graydon (1936). However, in the same year, Dolman and Wilson (1938) proposed the same name for staphylococcal enterotoxin. Very little is known about gamma toxin and, as there is still some confusion about its relationship

relationship with other staphylococcal toxins, no special effort was made to identify it in the animal strains which formed part of this survey.

Apart from what has been said there appears to be no mention, in the literature, of the presence of delta haemolysin in staphylococci of canine origin. In recent investigations by Richou et al (1956) and Rountree (1956) dog strains are still described as producing either alpha or beta toxins or a combination of these. Because of this and in view of the statement of Wilson and Harper (1947) that the presence of delta haemolysin was a more reliable indication of pathogenicity than even alpha toxin, and that delta haemolysin never occurred either alone or in coagulase negative strains, every effort was made, in this work, to identify accurately the haemolytic pattern of each strain. The results so obtained were correlated with the results of the coagulase test and, for confirmation, a selected number of strains were tested for lethal properties in mice and dermotoxin in rabbits.

The demonstration of staphylococcal haemolysins:

Strains were tested for haemolysins by the method of Elek and Levy (1950) who postulated that the patterns on blood agar could be explained by three haemolysins, alpha, beta and delta, occurring in seven different combinations.

Their technique is an adaptation of the Plate Method employing quantitative neutralisation by means of filter strips soaked in

in staphylococcal antitoxin, incorporated in the test medium.

In this work plates were prepared from rabbit, sheep and horse cells, the cells being washed three times in normal saline and resuspended to the original volume of the blood. Plates were poured by adding to each 12 ml. of a 1.5 per cent New Zealand agar nutrient broth base (pH. 7.4), 0.5 ml. of the prepared cells. Filter strips, approximately 60 x 15 mm. soaked in commercial staphylococcal antitoxin were sunk in each plate before the blood agar had set. The antitoxins used were products of the Wellcome Research Laboratories and had the following constitutions:-

	Antitoxin content/ml.		
	Alpha	Beta	Gamma
1. KCP 2296	510 units	250 units	not specified
2. RA 264A)			
3. RA 264A)			
4. RA 264A)	1250 units	not estimated	not estimated
5. RA 371A)			
6. RA 371A)			

In order to conserve sera the pattern of all strains was provisionally identified by stab inoculating sheep blood agar plates. After incubating the plates in air plus 20 per cent CO_2 for 48 hours, the strains were streaked on the surface of rabbit blood plates at right angles to the filter strips soaked in commercial staphylococcal alpha antitoxin. These were also incubated in air plus 20 per cent CO_2 for 48 hours.

Atypical strains and all strains showing alpha haemolysin were studied in detail using various sera, and blood plates prepared from horse, sheep and rabbit cells.

The haemolysins were identified as follows:-

Alpha toxin:

A colony of an alpha toxogenic staphylococcus on sheep blood agar was surrounded by a zone of complete lysis with an hazy indefinite margin. A similar effect was produced with rabbit blood but on horse blood lysis was indistinct. In addition to its haemolytic properties, true alpha toxin was shown to be both lethal and dermonecrotic.

Beta toxin:

This toxin, which is characteristic of most animal strains, was produced by both coagulase positive and coagulase negative strains. On sheep, bovine and goat blood plates it showed a large zone of discoloured cells surrounded by a sharp, well-defined margin. On cooling, the zone of partial lysis was replaced by complete haemolysis. Although beta toxin had no effect on horse cells, its behaviour with some rabbit bloods was found to be variable.

Delta haemolysin: showed as a small zone of complete lysis with a sharp margin surrounding the colony on sheep, horse and rabbit blood plates. As with alpha but not beta toxin, the effect was depressed or completely inhibited when the plates were incubated anaerobically. The delta haemolysin was non-lethal to white mice

mice but dermonecrotic to rabbits.

Other haemolysins:

A haemolysin, which closely resembled delta haemolysin was produced by a number of coagulase negative strains. Rabbit cells were especially sensitive to this haemolsyin which showed a synergistic action with beta toxin and was not inhibited by any of the antitoxins. This haemolysin is referred to as Epsilon haemolysin.

In addition to the staphylococci from infected ears and the ears, anterior nares and tonsils of healthy dogs, strains were also examined from other lesions in dogs and lesions of other animals. These latter strains would allow a comparison to be made not only of the haemolytic patterns but also of the individual toxins produced by staphylococci of different animal origins.

TABLE 98

The haemolysins produced by animal staphylococci

Coagulase Positive Strains

Origin of Strains	Number of strains.	Nature of the Haemolysin: Alpha	Beta	Delta	Other (or no) haemolysins
Dogs - infected ears	156	53	145	145	-
Healthy dogs - ears	36	10	29	35	-
nares	12	5	8	12	-
tonsils	4	2	2	4	-
Dogs - other lesions	19	6	18	19	-
Bovine lesions	42	30	37	37	-
Other animal lesions	13	10	8	13	-
Total	282	116	247	265	
All dog strains	227	76(34%)	202(89%)	215(95%)	-
All other animal strains	55	40(73%)	45(82%)	50(91%)	-

TABLE 99

The haemolysins of animal staphylococci

Coagulase Negative Strains

Source of Strains	Number of Strains	Nature of Haemolysin				No Haemolysis
		Alpha	Beta	Delta	Epsilon	
Dogs - infected ears	44	-	27	-	14	3
Healthy dogs - ears	48	-	7	2	20	21
nares	5	-	-	-	4	1
tonsils	7	-	-	-	5	2
Dogs - other lesions	4	-	1	-	2	1
Bovine lesions	8	-	1	-	2	5
Other animal lesions	2	-	-	-	-	2
	118	-	36	2	47	35
All dog strains	108	-	35(32%)	2(2%)	45(42%)	28(26%)
All other animal strains	10	-	1(10%)	-	2(2%)	7(70%)

These findings show that coagulase positive animal staphylococci are able to produce alpha, beta and delta haemolysins, but never epsilon haemolysin. Although Minett's (1936) observation that most animal strains produced beta toxin was confirmed, it was found that more pathogenic strains produced delta haemolysin than any other type of haemolysin. This shows that the correlation between coagulase and delta haemolysin production in human strains applies equally to staphylococci of animal origin. Similarly, the correlation between pathogenicity and the presence of alpha toxin applies equally to human and animal strains as all the alpha-producing strains were coagulase positive.

A comparison between dog and other animal pathogens showed that at least twice the number of "other animal" coagulase positive staphylococci produced alpha toxin as did dog strains. There was however, little difference between the staphylococci in their ability to produce beta and delta haemolysins.

The staphylococci from infected and healthy ears appeared to be similar, although some coagulase negative strains showed slight differences. The non-pathogenic infected ear strains mostly produced beta toxin only, whereas the healthy ear strains were usually non haemolytic or produced epsilon haemolysin. The two non-pathogenic ear strains which produced delta haemolysin did not do so alone as both strains also produced some beta toxin.

It has already been explained that the haemolytic patterns of human, and more especially animal, staphylococci have mostly been

been described as alpha, or beta or alpha and beta types. Although the above results are reproduced in a similar fashion in order to make for easier comparison with the findings of earlier workers, it must not be assumed that dog and other animal staphylococci generally produce a single haemolysin; indeed this was so with only 6 of 282 coagulase positive animal strains.

The non pathogenic strains gave very simple haemolytic patterns and when it is realised that of the 7 beta strains shown in the above Table, five produce only beta toxin and the other two strains beta and delta haemolysins, the haemolytic patterns of the coagulase negative strains will be obvious. As pathogenic staphylococci are capable of producing alpha, beta or delta haemolysins, it is theoretically possible for any one strain to produce haemolysins in one of seven possible combinations. The coagulase positive strains are so described in Table 100.

TABLE 100

The haemolytic patterns of coagulase positive staphylococci

Source of Strains	Number of Strains	Haemolysins						
		α.	αβ.	αβδ.	αδ.	βδ.	β.	δ.
Dogs - infected ears	156	3	6	36	8	101	2	-
Healthy dogs - ears	36	-	-	3	7	25	1	-
nares	12	-	-	1	4	7	-	-
tonsils	4	-	-	-	2	2	-	-
Other dogs - lesions	19	-	-	5	1	13	-	-
Bovine lesions	42	-	5	20	5	12	-	-
Other animal lesions	13	-	-	5	5	3	-	-
	282	3	11	70	32	163	3	-
All dog strains	227	3 (1%)	6 (3%)	45 (20%)	22 (10%)	148 (65%)	3 (1%)	- -
All other animal strains	55	-	5 (9%)	25 (45%)	10 (18%)	15 (27%)	- -	- -

The results in Table 100 show that individual haemolysins were produced by only 6 of the 282 coagulase positive staphylococci. Three of these showed alpha haemolysin and another three beta haemolysin. The fact that three strains produced beta haemolysin in the absence of either of the other two haemolysins is in itself unusual.

No coagulase positive strain produced delta haemolysin alone, although its presence, in combination with one or both of the other haemolysins, is probably of pathogenic significance.

Ten per cent and 18 per cent respectively of dog and other animal pathogenic strains produced both alpha and delta haemolysins. (Plate 33). This combination of haemolysins is not characteristic of canine strains and when it is realised that most of the aureus pigment-producing dog strains are of this type, it seems possible that the dogs from which these strains were recovered were, in fact, carriers of human or other animal staphylococci.

The most significant feature of this comparison of different animal staphylococci is perhaps the fact that most (65 per cent) of the dog pathogens produced beta and delta haemolysins with an undetectable amount, if any, of alpha toxin. (Plate 34). Most other animal coagulase positive strains (45 per cent) produced the full complement of haemolysins, whereas strains showing the beta delta pattern were only half as common.

At this point it should be emphasised that, although alpha-

PLATE 33.

Staphylococcal haemolysins.

Left.

Staphylococcus producing alpha haemolysin only.

Right.

Staphylococcus producing both alpha and delta haemolysins.

SHEEP blood agar plate, with surface strip soaked in Staphylococcus alpha antitoxin. Incubated in 20% CO_2 at 37° C. for 48 hours.

PLATE 34.

Staphylococcal haemolysins.

Showing the typical beta-delta haemolysin pattern of canine staphylococci.

SHEEP blood agar plate, with surface strip soaked in Staphylococcus alpha and delta haemolysins. Incubated in 20% CO_2 at 37°C. for 48 hours.

alpha-beta-delta strains (Plate 35), of all animals produced similar patterns on sheep blood agar, there is evidence to show that even such closely related staphylococci differ in certain respects, depending on the animal source of the strain. This will be referred to later.

The patterns produced by coagulase negative strains show that only two strains (2 per cent) produced delta haemolysins and then only in negligible amounts.

Perhaps of greater interest is the fact that 40 per cent of non pathogenic animal staphylococci and 39 per cent of dog strains produced a haemolysin on sheep blood plates which was synergistic with beta toxin and is referred to here as epsilon haemolysin. This factor differed from delta haemolysin which rarely occurs in non-pathogenic strains by not being dermonecrotic.

The non haemolytic, coagulase negative, staphylococci appeared to be of two types. The first type was unaffected by beta haemolysin but the other produced a factor which, by itself, had no effect but which, in the presence of beta haemolysin, produced small, circumscribed zones of complete haemolysis on sheep blood agar plates.

Having considered the correlation between pathogenicity and coagulase activity, the production of haemolysins, the liquefaction of gelatin and solid serum and the action on lactose, maltose and mannitol, it is now convenient to classify the results.

PLATE 35.

Staphylococcal haemolysins.

Left.

Weak alpha-toxin producing strain.

Right.

Typical animal staphylococcus, showing the alpha-beta-delta pattern.

SHEEP blood agar plate incorporating a filter strip soaked in Staphylococcus alpha and beta antitoxins. Incubated in 20% CO_2 at 37°C. for 48 hours.

The production of both alpha and beta haemolysins is inhibited by the antitoxins in the filter strip, whereas the delta haemolysin, which is not antigenic, still shows up to and beyond the filter strip.

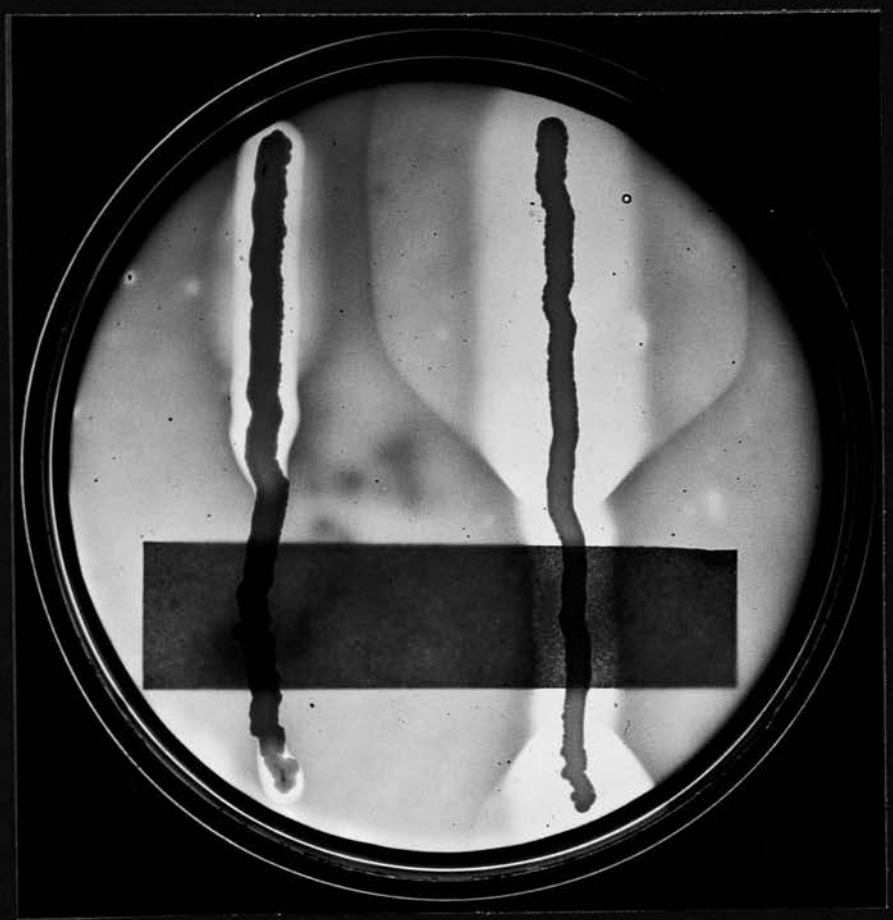


TABLE 101.

Classification of staphylococci of animal origin.

Coagulase positive strains.

Source of strains and numbers producing the effects shown				Haemolysin	Haemolysin	Haemolysin	Gelatin liquefied	Solid serum liquefied	Lactose	Maltose	Mannitol
4.	3.	2.	1.	α.	β.	δ.					
24	3	2	16	+	+	+	+	+	+	+	+
1	1	2	17	+	+	+	+	+	+	+	0
0	0	0	1	+	+	+	+	+	+	0	0
3	1	0	1	+	+	+	+	0	+	+	+
0	0	0	1	+	+	+	0	+	+	+	+
0	0	0	1	+	0	0	+	+	+	+	+
0	0	0	1	+	0	0	+	0	+	+	+
0	0	0	1	+	0	0	+	0	+	0	0
8	0	9	3	+	0	+	+	+	+	+	+
0	0	4	2	+	0	+	+	+	+	+	0
0	0	0	1	+	0	+	0	+	0	0	+
2	1	0	2	+	0	+	+	0	+	+	+
2	0	0	2	+	+	0	+	+	+	+	+
0	0	0	1	+	+	0	+	+	+	+	0
0	0	0	3	+	+	0	+	0	+	+	+
3	5	12	58	0	+	+	+	+	+	+	+
0	6	19	34	0	+	+	+	+	+	+	0
0	1	0	5	0	+	+	+	+	+	0	+
1	1	2	1	0	+	+	+	+	+	0	0
0	0	0	1	0	+	+	+	+	0	0	+
0	0	0	1	0	+	+	+	+	0	0	0
0	0	0	1	0	+	+	+	0	+	0	+
0	0	0	1	0	+	0	+	+	+	+	0
0	0	1	1	0	+	0	+	+	+	0	+
10	0	0	0	0	+	+	+	0	+	+	+
1	0	0	0	0	+	+	0	+	+	0	+
0	0	1	0	0	+	+	+	+	0	+	0
55	19	52	156								

Legend: 1. = Dogs - infected ears
2. = Healthy dogs - ears, nares and tonsils
3. = Other lesions in dogs.
4. = Lesions in other animals

TABLE 102.

The characters of staphylococci of animal origin.

Coagulase negative strains.

Source of strains and numbers producing the effects shown.				Haemolysin α.	Haemolysin β.	Haemolysin δ.	Haemolysin or no haemolysin ε.	Gelatin liquefied	Solid serum liquefied	Lactose	Maltose	Mannitol
4.	3.	2.	1.									
		2			+	+		+	+	+	0	0
			2		+			+	+	+	+	+
1		4	1		+			+	+	+	+	0
			6		+			+	+	+	0	+
			1		+			+	+	0	+	+
			1		+			+	+	0	0	0
	1	1	4		+			+	+	0	0	+
			2		+			+	0	+	+	+
			2		+			+	0	+	+	0
			1		+			+	0	0	0	0
			3		+			0	+	+	+	0
			1		+			0	+	+	0	+
			1		+			0	+	0	0	0
			2		+			0	0	+	+	+
		1	4				+	0	0	+	+	+
		7	7				+	0	0	+	+	0
			1				+	0	0	+	0	0
		1	1				+	0	0	+	0	+
		1	1				+	0	0	0	+	+
1		4	1				+	+	0	0	0	0
		8	1				+	0	0	0	+	0
		4	1				+	0	0	+	+	0
2		2					+	+	+	+	+	+
4	2	2					+	+	+	+	+	0
	1						+	+	+	+	0	0
		1					+	+	+	0	+	0
		3					+	+	+	0	+	+
		1					+	+	+	0	0	0
		6					+	+	0	+	+	+
1		1					+	+	0	+	0	+
1							+	+	0	0	0	+
		2					+	+	0	+	+	0
		2					+	+	0	0	+	0
		7					+	0	0	0	0	0
10	4	60	44									

Legend: 1. = Dogs - infected ears
2. = Healthy dogs - ears, nose and tonsils
3. = Other lesions in dogs
4. = Lesions in other animals

The nature of the haemolysins produced by staphylococci of canine origin

The effects of the various haemolysins on blood agar plates have been adequately described by Elek and Levy (1950), Marks (1951) and others. However, since Smith (1947), Gustaffson (1954) and Richou (1956) have reported that most dog staphylococci produce alpha or beta toxins, or a combination of these whereas in this work very few were found to be of this pattern, it is necessary to consider in some detail the nature of the haemolysins of canine staphylococci.

The initial step in the identification of the haemolysins was by stab inoculation of sheep blood agar plates with an overnight agar-slope culture of the staphylococcus. The plates were incubated for 48 hours at 37°C. in an atmosphere of 20 per cent CO_2 when evidence of beta toxin was obtained by a zone of incomplete haemolysin, with a well-defined margin surrounding the colony. A small zone of complete lysis with a definite margin immediately surrounding the colony suggested the presence of an additional lysin, the delta haemolysin. Strains which produced a much larger area of complete haemolysis with a hazy margin but still within the beta toxin zone were provisionally identified as alpha-beta or alpha-beta-delta types. Where the same effect was produced, in the absence of beta toxin, the staphylococcus was presumed to be an alpha or an alpha-delta strain. The full pattern of all such strains was established by streaking the

the cultures on the surface of rabbit blood plates at right angles to a filter strip soaked in commercial staphylococcus antitoxin which was incorporated in the medium. (Plate 36). This plate shows the routine method of identification of beta delta strains. No beta effect is visible and the clear narrow zones of haemolysis which are unaffected by the anti-haemolysins in the filter strip are due to the production of non-antigenic haemolysins, the delta haemolysins.

Cultures producing alpha or alpha and delta haemolysins in addition are readily identified by the same methods. (Plate 37).

The various combinations of haemolysins produced by dog staphylococci occurred in the following order of frequency - beta-delta, alpha-beta-delta, alpha-delta, alpha beta, alpha and beta, examples of which are shown in Plates 33 - 37 and 47 - 48. For confirmation, the lethal effect and the ability to produce skin necrosis of a selected number of strains were investigated biologically. Details of these experiments are given later. For the present, it is sufficient to say that alpha and alpha delta strains showed lethal properties and were dermonecrotic, but the alpha-beta-delta and beta-delta strains were dermonecrotic but not lethal.

This apparent loss of virulence of the dog staphylococci which nevertheless produced the full complement of haemolysins was all the more remarkable when it was shown that similar organisms from cattle and sheep were highly lethal to white mice.

PLATE 36.

Staphylococcal haemolysins.

Left.		Right.	
Strain 1.	Strain 2.	Strain 3.	Strain 4.
Beta-delta haemolysins. (Coagulase) +ve.	Beta-delta haemolysins. (Coagulase) +ve.	Epsilon haemolysin. (Coagulase) -ve.	Beta-delta haemolysins. (Coagulase) +ve.

RABBIT blood agar plates incorporating filter strip soaked in Staphylococcus alpha antitoxin. Incubated in 20% CO_2 at 37°C. for 24 hours only.

This method was used routinely to confirm the presence of delta haemolysin in beta-delta producing strains, the strains having been previously tested by stab inoculation on sheep blood agar plates.

PLATE 37.

Staphylococcal haemolysins.

Left. Right.

Strain. 1.	Strain 2.	Strain 3.	Strain 4.
Alpha-delta haemolysins.	Alpha haemolysin only.	Beta haemolysin only.	Alpha-delta haemolysins.

RABBIT blood agar plate incorporating a filter strip soaked in Staphylococcus alpha antitoxin. Incubated in 20% CO_2 at 37°C. for 24 hours only.

This method was used to differentiate alpha and alpha-delta producing strains, a number of which also formed beta toxin on SHEEP blood agar plates.

It will be noticed that strains 1, 2, and 4 are producing different amounts of alpha toxin, judging by the distance of the haemolytic zones from the filter strip. Beta toxin usually has no haemolytic effect on rabbit cells.

This suggested that, not only was the alpha toxin of alpha-beta-delta dog and other animal staphylococci different, but that it differed from the alpha toxin of "dog alpha" or "dog alpha delta" strains.

It will be recalled that Minett (1936) suggested that dog strains produce little if any alpha toxin. That this was the reason for the low virulence of the staphylococci in question was apparently confirmed by the behaviour of one of the dog strains (Strain 46). This strain produced, in addition to beta and delta haemolysin, an extremely low concentration of alpha toxin (plate 38). A number of other strains that produced similar patterns when stab inoculated on sheep blood agar gave an entirely different effect with sheep blood agar containing antitoxin impregnated strips (Plate 39).

This plate showed that there was only limited neutralisation of the haemolysins by the alpha antitoxin in the filter paper strip. (1,200 units/ml.). A filter strip soaked in antisera containing 510 units and 250 units respectively of alpha and beta antitoxins was sunk in a blood plate prepared from the same suspension of sheep cells. The effect produced (Plate 40) confirmed the presence of beta toxin but not alpha or delta. An unusual haemolytic zone was seen between the lower borders of the neutralised beta zone and the filter strip. This almost completely haemolytic area, with an indefinite margin, resembled the action of alpha toxin on sheep red cells, but as there was no

PLATE 38.

Staphylococcal haemolysins.

Showing Strain 46 which produced beta-delta haemolysins and a small amount of alpha haemolysin.

SHEEP blood agar plate with a surface strip soaked in Staphylococcus alpha antitoxin. Incubated in 20% CO_2 at 37°C. for 48 hours.

Few coagulase positive canine strains produced appreciable amounts of alpha toxin. In a few beta-delta strains, however, there was evidence of traces of a third haemolysin which lysed both SHEEP and RABBIT blood. This effect was thought to be due to small amounts of typical alpha toxin or to a new haemolysin which it closely resembled. (See text.)

PLATES 39 & 40.

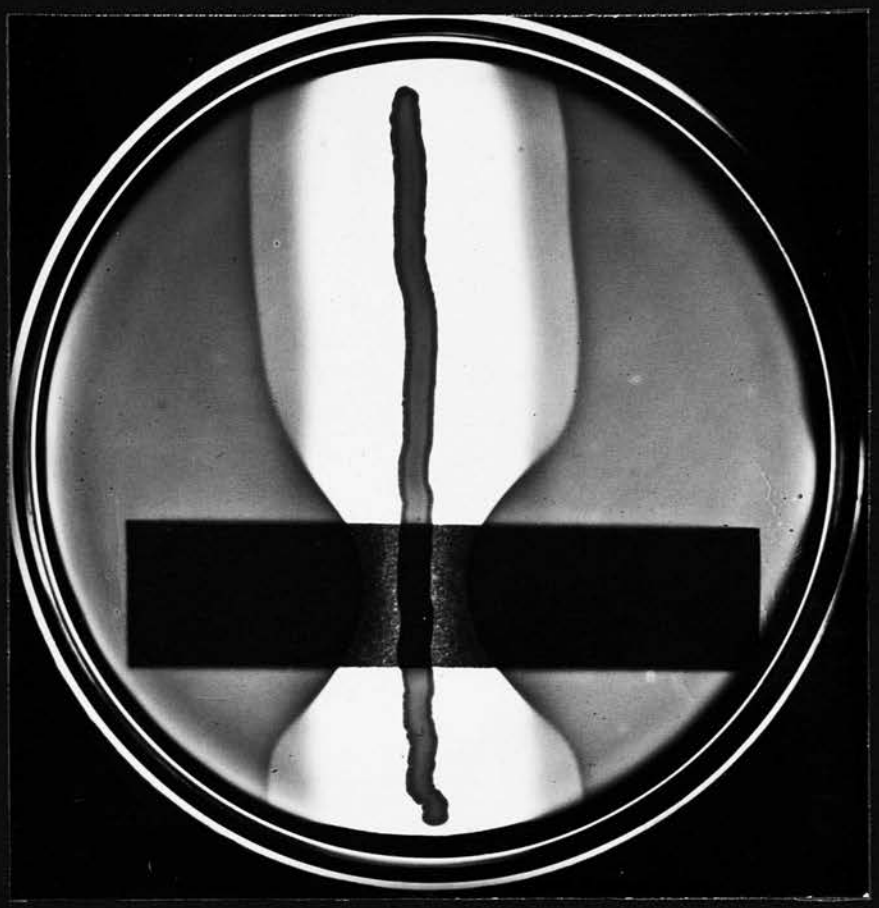
Staphylococcal haemolysins.

Plate 39 Showing Strain 201 streaked on a SHEEP blood agar plate with a surface strip soaked in alpha antitoxin.

Plate 40 The same strain (201) on a SHEEP blood agar plate incorporating a filter strip soaked in Staphylococcus alpha and beta antitoxins.

The blood agar plates were both incubated in 20% CO_2 at 37°C. for 48 hours.

All three haemolysins (alpha, beta and delta) are shown in Plate 39. By streaking the same strain across a filter strip soaked in antitoxin with a high concentration of beta antitoxin a fourth haemolysin was seen, between the filter strip and the beta and alpha haemoltic zones. (Plate 40).



no evidence of neutralisation by the anti-haemolysins, it was either a type of alpha toxin, antigenically distinct from the usual toxin, or some other unidentified toxin.

Three of these atypical strains were streaked on rabbit blood plates incorporating alpha-beta antitoxin strips, with a number of control strains (Plate 41). The results obtained were quite unexpected in that zones of partial haemolysis were produced which were characteristic of neither alpha, beta, nor delta haemolysins.

Contrary to the general opinion that rabbit red cells are unaffected by beta toxin, the majority of beta-producing dog strains did occasionally show evidence of very weak haemolysins when tested against the red cells of certain rabbits (Plate 42). The effect was invariably minimal and did not resemble the unidentified haemolysins of Plate 40. Moreover, as the beta toxin produced by the four beta-delta control strains failed to influence the rabbit cells, it is unlikely that the effect shown is due to beta haemolysin.

The presence of flocculation lines precludes the possibility of it being a delta type haemolysin.

The only other common haemolysin that affects rabbit cells is alpha toxin and, while it is obvious that the haemolytic effect here described is not due to a typical alpha toxin producing staphylococcus, a number of features suggest that it may be a type of alpha toxin.

The tests were repeated using red cells from another rabbit,

PLATE 41.

Staphylococcal haemolysins.

Key to the strains.

64	201	162	165
F I L T E R S T R I P.			
199	198	191	177

64 = alpha-delta strain.

177 = epsilon strain.

162, 165, 191 = beta-delta strains.

198, 199, 201 = beta-delta strains plus new haemolysin.

RABBIT blood agar plate with a surface strip soaked in Staphylococcus alpha and beta antitoxins. Incubated in 20% CO_2 at 37°C. for 24 hours only.

Note the degree of haemolysis with the three strains, 198, 199 and 201, compared with the typical alpha haemolytic zone of strain 64. The faint flocculation lines (shown by arrows) suggest that the unidentified haemolysin is only partially neutralised by the antitoxins in the filter strip.

PLATE 42.

Staphylococcal haemolysins.

Key to strains.

170	169	168	167
F I L T E R		S T R I P.	
166	165	164	163

163 168
164 169 = beta-delta
165 170

166
167 = alpha-beta-delta

RABBIT blood agar plate with surface strip soaked in Staphylococcus alpha and beta antitoxins. Incubated in 20% CO_2 at 37°C. for 24 hours only.

To demonstrate the partial haemolysis of the red cells of certain rabbits by beta-toxin producing staphylococci of canine origin.

Note that some strains produce more delta haemolysin than others.

rabbit, and filter paper strips soaked in alpha antitoxin. The plates were incubated at 37°C. in air plus 30 per cent CO_2 for 24 hours when they were examined and reincubated for a further 24 hours. A staphylococcus which produced both alpha and delta haemolysins was used as a control on each plate. After 24 hours' incubation the plates (Plates 43 and 44) showed the same zones of partial haemolysis surrounded by a dark hazy margin which extended on either side beyond the flocculation lines. A day later these zones were intensified but the dark borders had disappeared leaving the partially haemolytic zones with ill-defined margins (Plates 45 and 46).

The results of a similar experiment using surface strips soaked in staphylococcus alpha and beta antitoxins, instead of alpha antitoxin, are shown in Plate 47.

Morgan and Graydon (1936) considered that there were two antigenically distinct alpha toxins which they called alpha-one and alpha-two. Kojima and Kodama (1939), on the other hand, have postulated three alpha lysins, namely alpha-one, two and three.

Whether or not the alpha toxin of animal staphylococci consists of a mixture of monovalent lysins is not known but the above results suggest that such might be the case in a number of dog strains.

The properties of this unidentified haemolysin may be summarised as follows:-

1. Produces zones of partial haemolysis with an indefinite

PLATES 43 and 44.

Staphylococcal haemolysins.

Plate 43. Left. Right.

Strain 64. Alpha-delta (control)

Strain 201 (unidentified).

Plate 44. Left. Right.

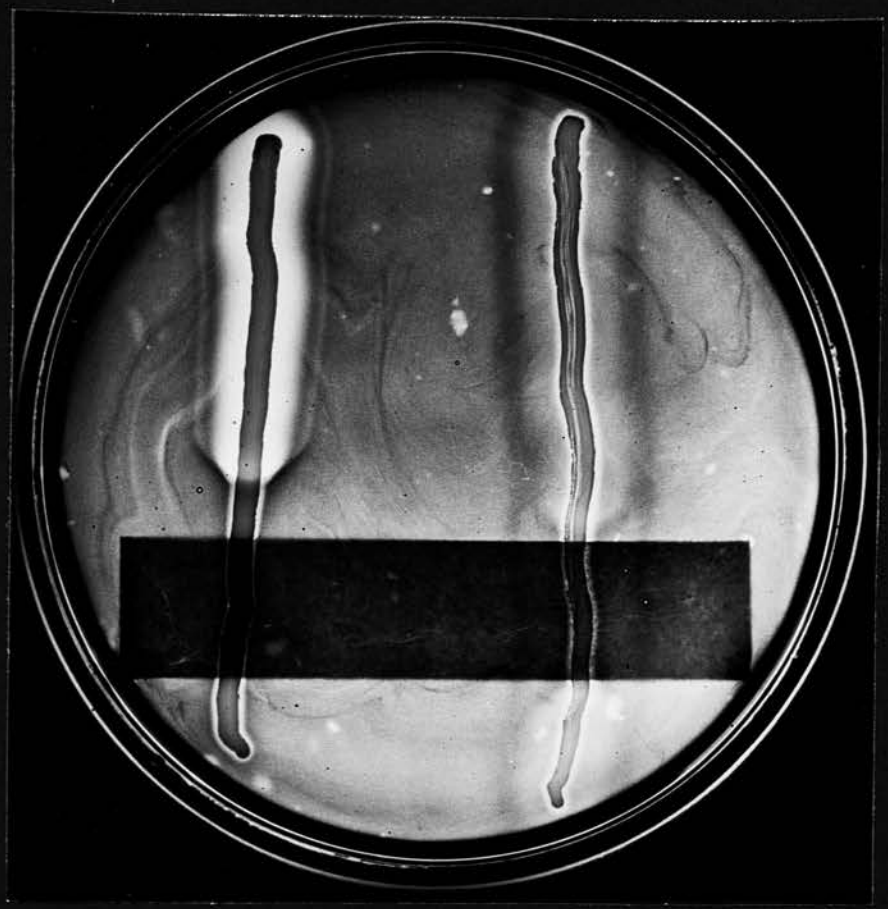
Strain 198 (unidentified)

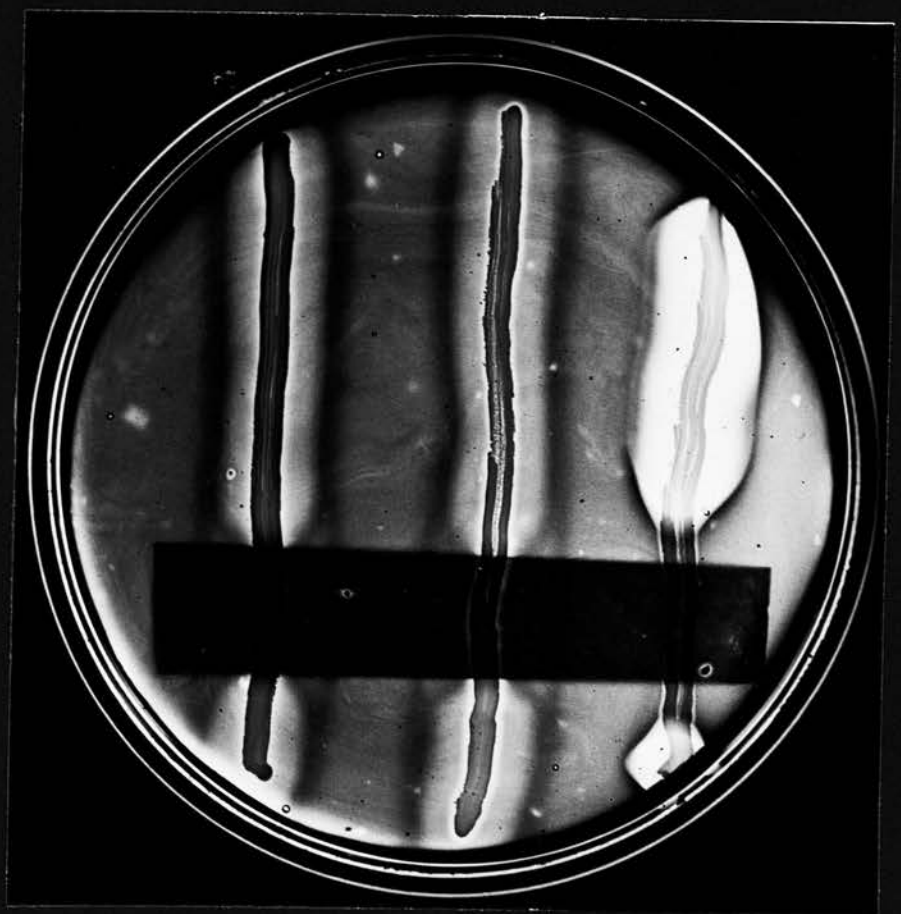
Strain 199 (unidentified)

Strain 64. Alpha-delta (control)

RABBIT blood agar plates incorporating filter strips soaked in Staphylococcus alpha antitoxin. Incubated in 20% CO_2 at 37°C. for 24 hours only.

Note the indefinite margins of the haemolytic zones of the unidentified strains and the incomplete neutralization by the alpha antitoxin.





PLATES 45 and 46.

Staphylococcal haemolysins.

Showing the same cultures as in Plates 43 and 44 after a further 24 hours incubation in 20% CO_2 at 37°C.

Note that the haemolytic effects of the unidentified haemolysin are now neutralised by the alpha antitoxin impregnated filter strip.
The unidentified haemolysin fails to completely lyse the rabbit red cells, as does the alpha toxin producing control strain, but has a more marked effect than beta toxin.

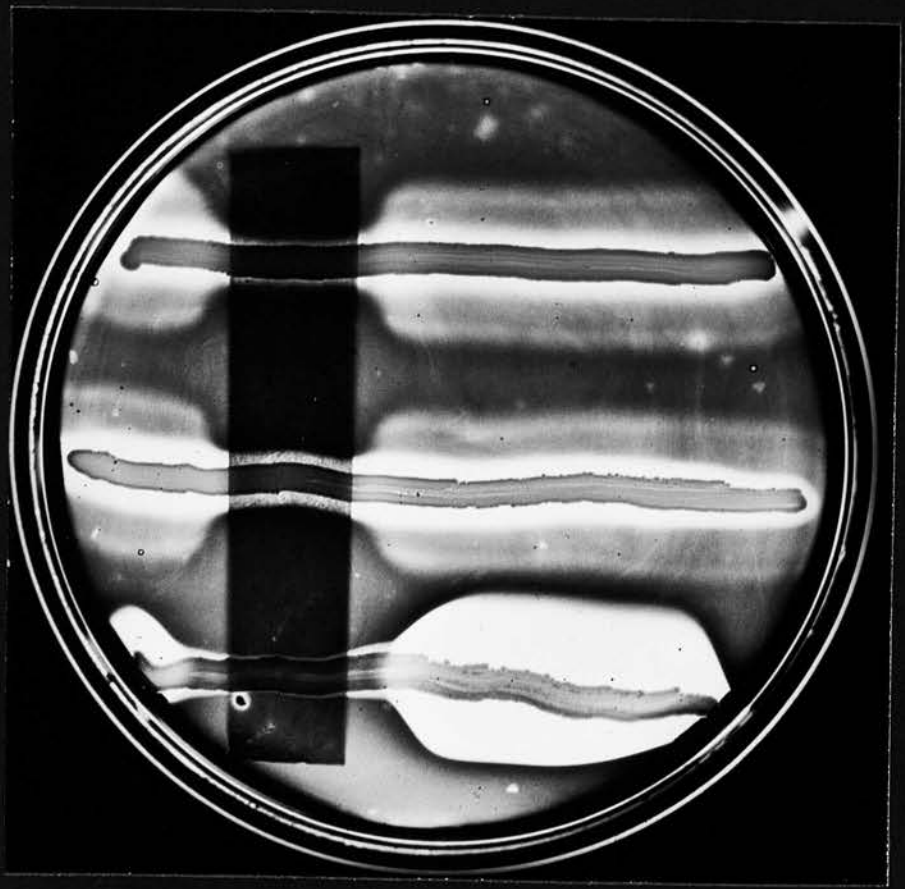


PLATE 47.

Staphylococcal haemolysins.

Left. Right.

Coagulase negative control strain.	Coagulase positive	Coagulase positive control strain.
Beta haemolysin only.	Unidentified strain (201).	Alpha-delta haemolysins.

RABBIT blood plate with surface strip soaked in alpha and beta antitoxins. Incubated in 20% CO_2 at 37°C. for 24 hours only.

To demonstrate the neutralising action of alpha and beta antitoxins on known beta, known alpha-delta and an unidentified haemolysin producing strain, on rabbit red cells.

Even after 24 hours there is evidence of both beta and delta haemolysins being produced by the test strain. The presence of an unidentified haemolysin which is not completely neutralised by either antitoxin is confirmed.

indefinite margin on sheep and rabbit blood plates.

2. Is incompletely neutralised by commercial alpha antitoxin
3. Is produced only by coagulase positive staphylococci
4. Has no lethal effects
5. Is distinct from alpha, beta and delta haemolysins

Additional characteristics of animal staphylococcal haemolysins

Marks (1952) demonstrated the interaction between the three common haemolysins on different species of blood agar plates. On sheep blood plates the beta toxin depressed the effect of alpha toxin but was synergistic with delta haemolysin. The combination of alpha toxin and delta haemolysin was 'additive'.

This opportunity was taken to study the interaction between haemolysins of canine staphylococci.

Sheep and rabbit blood agar plates were inoculated by streaking overnight agar slope cultures on the surface of the medium so that two strains were drawn at right angles through the third strain. The plates were incubated at 37°C. in air plus 30 per cent CO_2 for two-24 hour periods.

Sheep blood:

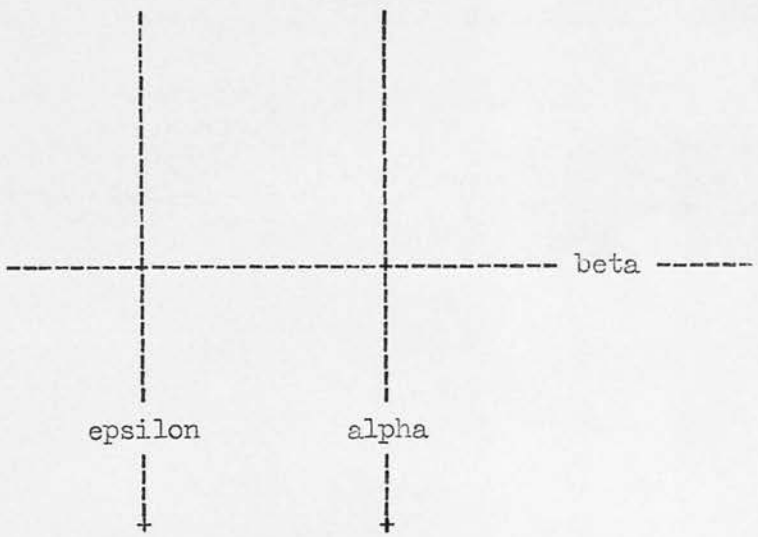
Beta and alpha toxins:

After 24 hours (Plate 48) it was seen that the interaction of alpha toxin in the presence of weak concentrations of beta toxin was slightly synergistic, whereas in higher concentrations of beta toxin the alpha effect was depressed. At 48 hours

PLATES 48 and 49.

Staphylococcal haemolysins.

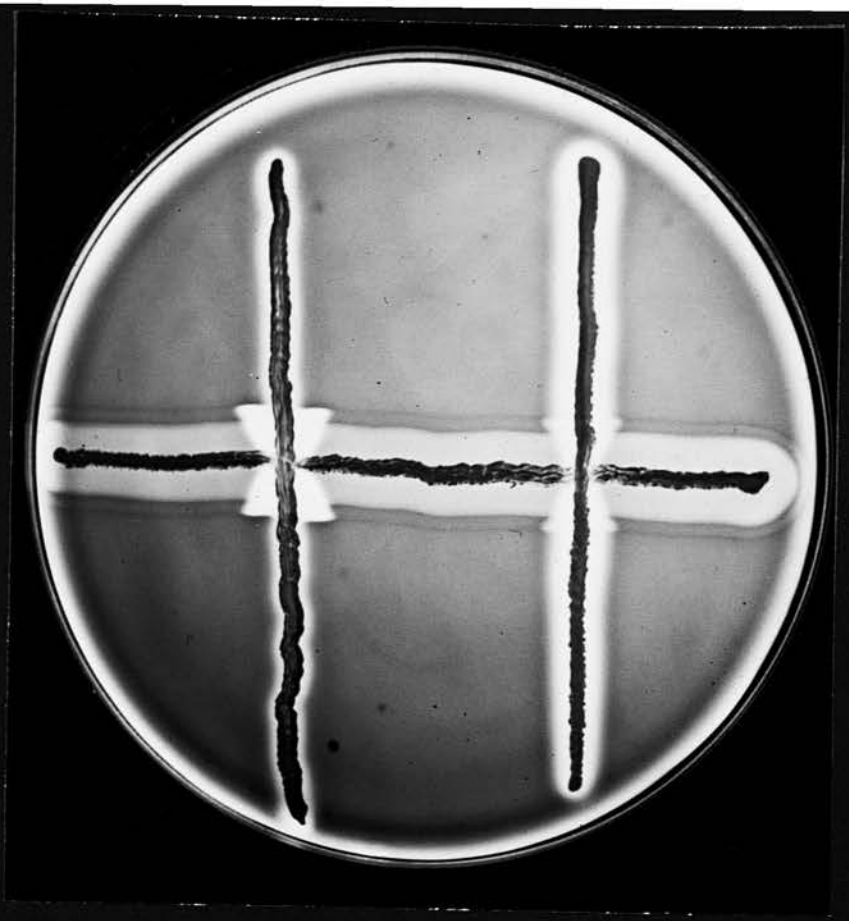
Key to plates.

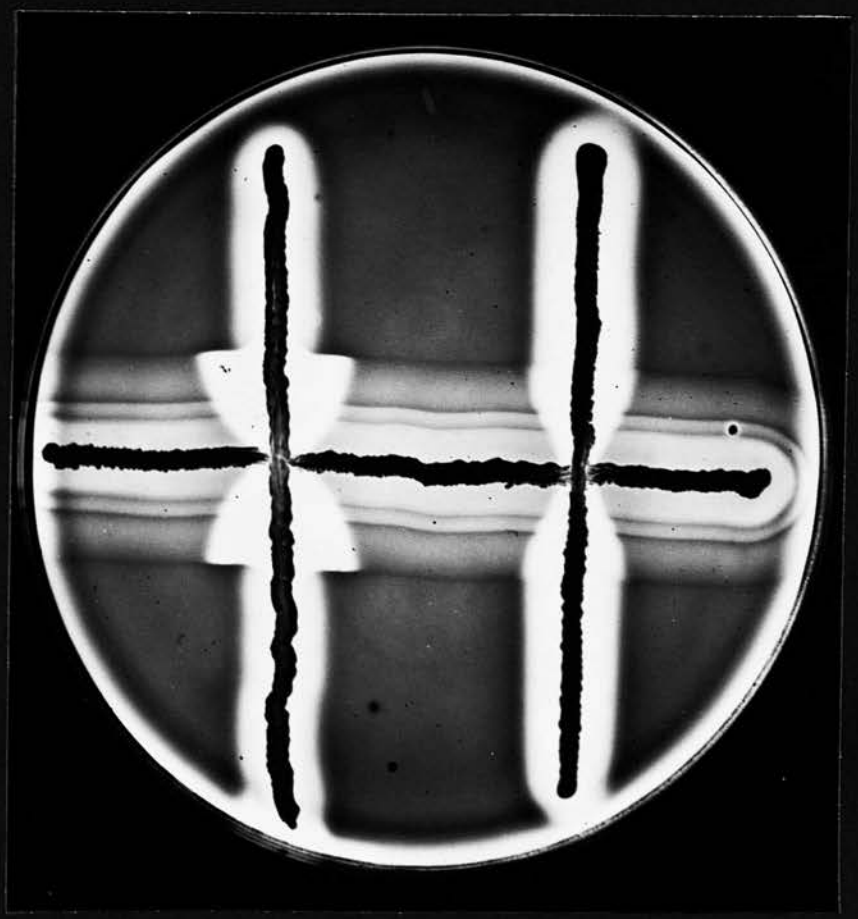


SHEEP blood agar plates incubated in 20% CO_2 at 37°C.
for 24 hours (Plate 48) and a further 24 hours (Plate 49).

Showing the combined effects of staphylococcal haemolysins.

Note that the combined haemolytic effect of beta and epsilon haemolysins is synergistic in lower concentrations of beta toxin, whereas the effect of alpha toxin is inhibited by beta toxin.





hours (Plate 49) there was little evidence of synergism in low concentrations of beta toxin but the inhibitory effect was maintained.

Beta and epsilon toxins:

On the same plates it will be seen that the susceptibility of sheep cells to the haemolysin of a coagulase negative staphylococcus is increased in the presence of small amounts of beta toxin but that the effect is diminished in higher concentrations of beta toxin.

Rabbit blood:

Beta and alpha toxins:

That the effects of alpha toxin on rabbit cells are also depressed by beta toxin is shown in Plates 50 and 51.

Alpha toxin and delta haemolysin:

The same plate (Plate 50) shows the 'additive' effect of these two haemolysins on rabbit blood.

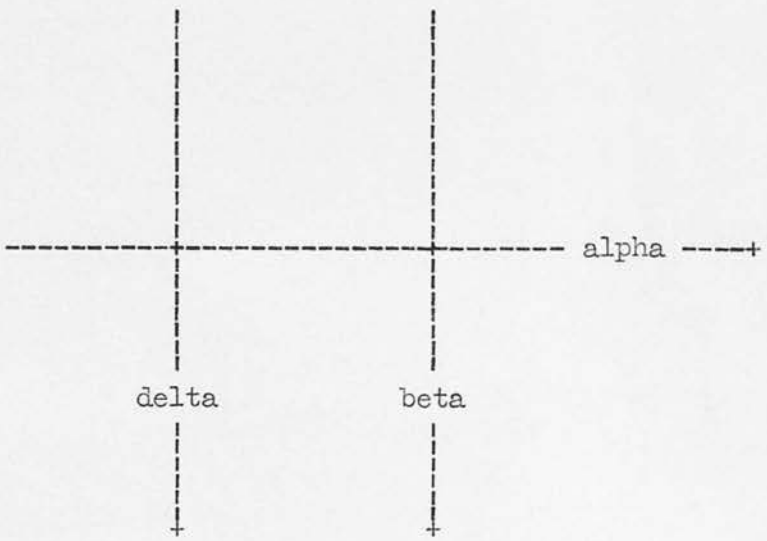
Beta toxin and epsilon toxin:

The same coagulase negative staphylococcus was used as in the experiments shown on Plates 48 and 49. On sheep blood agar the interaction between beta toxin and epsilon toxin was synergistic, whereas on rabbit blood agar, the effect appears to be reversed. Plate 51 shows that on rabbit blood agar there is no evidence of synergism in the area of weak beta toxin but when the epsilon producing strain approaches high concentrations of beta

PLATE 50.

Staphylococcal haemolysins.

Key to plate.



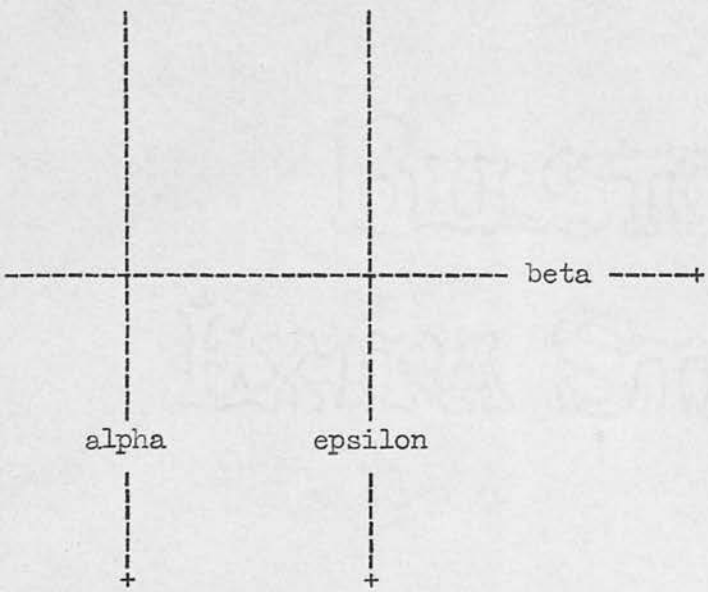
RABBIT blood agar plate incubated in 20% CO_2 at 37°C. for 24 hrs. Showing the interaction of staphylococcal haemolysins on rabbit red cells.

NOTE that the haemolytic effect of alpha toxin is diminished in the vicinity of the beta toxin producing strain, although beta toxin has no lytic effect on rabbit red cells. The combined effect of alpha and delta haemolysins is neither synergistic nor antagonistic, the wider haemolytic zone (bottom left) being due to a 'carrying over' of the alpha producing strain during inoculation of the plate.

PLATE 51.

Staphylococcal haemolysins.

Key to plate.



RABBIT blood agar plate incubated in 20% CO_2 at 37°C. for 24 hrs.

Showing the interaction of staphylococcal haemolysins on rabbit red cells.

NOTE that the haemolytic effect of alpha toxin is again depressed by beta toxin. In this experiment the coagulase negative epsilon producing staphylococcus failed to haemolyse the rabbit red cells in the presence of beta toxin, whereas on sheep blood agar the combined haemolytic effect of beta and epsilon haemolysins was synergistic. (Plates 48 & 49).

beta toxin (in the immediate vicinity of the beta culture) the haemolytic properties of the epsilon haemolysin is completely depressed. No visible haemolysis is seen during the "carry over" of some of the beta-producing organisms but when these have been "left behind" the epsilon haemolysin is able, once more, to haemolyse the rabbit cells.

Lethal effect:

The lethal effect of a number of selected strains was studied in order to confirm the ability of a small proportion of dog staphylococci to produce alpha toxin. Smith (1947) reported that 23 of 24 coagulase positive dog staphylococci produced both alpha and beta toxins, none of which was lethal to mice. Overnight nutrient broth cultures were centrifuged and the centrifugate pipetted off and retained. The deposited bacteria were washed twice and resuspended in sterile normal saline to the original volume. Of this toxin-free bacterial suspension 0.3 ml was inoculated intravenously into each of three white mice. Surviving mice were examined over a period of eight weeks.

The 44 strains which were examined produced various haemolysins

4	strains produced	alpha toxin only
4	" "	alpha and beta toxins only
4	" "	alpha and delta toxins only
8	" "	all three haemolysins
12	" "	beta and delta haemolysins
5	" "	beta toxin only
5	" "	none of the three common haemolysins

In addition, 2 sheep staphylococci which produced all three haemolysins were included as controls.

A post-mortem examination was carried out on all the mice that died. The kidneys were examined for evidence of pyaemic abscesses and cultures were taken from both kidneys and heart blood. Cultures from all mice dying within ten days showed a heavy growth of staphylococci.

The mice which survived for eight weeks were killed and the kidneys examined for evidence of macroscopic lesions.

The results of this experiment are shown in Table 103.

TABLE 103

The lethal effect of staphylococci on mice

Number of Strains	Haemolysins Produced	Number of Lethal Strains	Number of Mice (Death in Days) 1	2	8	8	Total Mice Died/Inoc.	Total Kidneys With lesions/Examined
4	α.	4	3	1	7	1	12/12	23/24
4	αδ.	4	2	5	5	-	12/12	16/24
4	αβ.	1	-	-	-	2	2/12	3/12
8	αβδ.	0	-	-	-	-	0/24	7/30
* 2	αβδ.	2	4	2	-	-	6/6	
12	βδ.	0	-	-	-	-	0/36	11/72
5	β.	0	-	-	-	-	0/15	7/30
5	[illegible].	0	-	-	-	-	0/15	2/30

NOTE: * = Sheep strains
All other strains are of canine origin

The lethal effect of coagulase positive dog staphylococci is most pronounced in strains which produce alpha toxin in the absence of beta toxin. When beta toxin is also present the lethal effect is virtually lost. This may be due to the fact that (1) few beta toxin strains are capable of producing detectable amounts of alpha toxin, (2) dog strains produce so much beta toxin that the alpha toxin effect is inhibited, or (3) the alpha toxin present in beta-producing dog strains is different from the typical lethal alpha toxin of human and other animal staphylococci.

The two sheep strains (alpha-beta-delta types) were both lethal to mice.

Macroscopic kidney lesions were seen in about a third of the mice that survived for eight weeks.

Dermotoxin:

The same strains that were tested for lethal effects were examined for dermotoxin. The undiluted supernatants from the overnight nutrient broth cultures (37°C. in air plus 30 per cent CO_2) were checked for purity and injected in 0.1 ml. amounts, intradermally into white depilated rabbits. The rabbits were examined daily for four days.

The results are given in Table 104.

TABLE 104

Dermonecrotic toxin produced by animal staphylococci

Number of Strains	Haemolysins Produced	Number of Strains Producing the Reaction		
		Negative	Weak	Strong
4	α.	-	1	3
4	αδ.	-	2	2
4	αβ.	1	2	1
8	αβδ.	-	1	7
* 2	αβδ.	-	-	2
12	βδ.	-	3	9
5	β.	4	1	-
5	ε. ∞ -.	5	-	-

NOTE: * = Sheep strains
All other strains are of canine origin

These results show that fluid cultures of canine staphylococci may contain a dermonecrotic toxin which is capable of producing skin necrosis in rabbits.

The effect is usually confined to strains which produce alpha toxin or delta haemolysin alone or in combination with other haemolysins. Coagulase negative staphylococci and strains producing beta toxin only do not form dermotoxin.

Production of pigment:

The ability of staphylococci to produce pigment is no longer a reliable indication of pathogenicity. Although most human and animal pathogenic strains produce the typical gold pigment, the majority of dog strains were unable to produce pigment, irrespective of their coagulase activity. Smith (1947) found only three gold pigment producers in twenty-four coagulase positive dog strains, in contrast to Richou's (1956) figure of 39 per cent.

On the other hand, Jones (1955) reported that "S. pyogenes var aureus" was present in 34 specimens from 78 dogs and was predominant in lesions in the dog.

In this work pigment production was studied on a variety of media including nutrient agar, maltose agar, blood agar and solid serum slopes. Finally, all strains were re-examined on milk agar after the method of Fujita and Yoshioka (1938). The inoculated plates were incubated at 37°C. overnight, examined and reincubated for a further 24 hours. They were then left at room temperature for five more days and re-examined. Although these tests were done primarily to study pigment production, many strains showed a translucent zone of clearing around the colony due, presumably, to hydrolysis of the casein. On the surface of the plate an iridescent zone appeared which increased in diameter as incubation progressed. Frequently, both this action of staphylococcal lipase on milk fat and the clear zone of casein hydrolysis were obtained by the same strain. The ability of a

a strain to hydrolyse casein was not enhanced by the addition of 30 per cent CO_2 to the atmosphere, nor was it inhibited when the cultures were incubated anaerobically. Although there was much gradation of pigment in the colonies on milk agar, e.g. white, off-white, new ivory, old ivory, and grey, it will be more convenient to describe them in the accompanying Tables as white, grey or aureus. In addition, some of the coagulase negative strains will be called yellow pigment producers to include lemon and canary yellow varieties.

TABLE 105

The action of staphylococci on milk agar

Coagulase Positive Strains

Source of Strains	Number of Strains	Pigment Produced			Hydrolysis of Casein	
		White	Grey	Aureus	(+)	(-)
Dogs - infected ears	156	81	84	14	133	23
Healthy dogs -						
ears	36	9	20	7	29	7
nares	12	1	6	5	9	3
tonsils	4	0	2	2	3	1
Dogs - other lesions	19	5	12	2	17	2
Bovine lesions	42	6	2	34	12	30
Other animal lesions	13	2	2	9	7	6
	282	81	128	73	210	72
All dogs	227	73 (32%)	124 (55%)	30 (13%)	191 (84%)	36 (16%)
All other animals	55	8 (15%)	4 (7%)	43 (78%)	19 (35%)	36 (65%)

TABLE 106

The action of staphylococci on milk agar

Coagulase Negative Strains

Source of Strains	Number of Strains	Pigment Produced: White	Grey	Aureus	Yellow	Hydrolysis of Casein: (+)	(-)
Dogs - infected ears	44	30	14	-	-	22	22
Healthy dogs - ears	48	26	7	10	5	12	36
nares	5	4	0	1	-	-	5
tonsils	7	5	1	1	-	-	7
Dogs - other lesions	4	4	-	-	-	1	3
Bovine lesions	8	3	2	3	-	1	7
Other animal lesions	2	-	-	2	-	1	1
	118	72	24	17	5	37	81
All dogs	108	69 (64%)	22 (20%)	12 (11%)	5 (5%)	35 (32%)	73 (69%)
All other animals	10	3 (30%)	2 (20%)	5 (50%)	-	2 (20%)	8 (80%)

The inability of pathogenic dog staphylococci to produce aureus pigment is shown by the fact that 87 per cent of coagulase positive dog strains produced colonies that were either white or grey, whereas only 22 per cent of other animal pathogenic

pathogenic staphylococci did so. Aureus pigmented colonies were given by 78 per cent of animal strains as against 13 per cent of dog pathogens.

Dog strains (67 per cent) hydrolysed milk casein more readily than other animal staphylococci (32 per cent) irrespective of their ability to produce coagulase.

Nitrate Reduction:

Kauffman's (1951) method was used to study the ability of staphylococci to reduce nitrates.

Although all but 8 of 118 strains reduced nitrates to nitrites, there was no correlation between nitrate reduction, pathogenicity or the species of animal from which the strains were derived. These results compare favourably with the figure of 97 per cent obtained by Shaw et al (1951) from their study of coagulase positive strains from a variety of sources.

Voges-Proskauer reaction:

Barritt's (1936) method was preferred for demonstrating the production of acetylmethylcarbinol by 111 strains of pathogenic and non pathogenic animal staphylococci.

TABLE 107

	Coagulase Positive V.P.		Coagulase Negative V.P.	
	+	-	+	-
Dog otitis	12	35	5	6
Dog other lesions	5	3	14	1
Bovine lesions	9	1	2	3
Other animal lesions	7	6	1	1
	33	45	22	11

Most (69 per cent) other animal pathogenic staphylococci gave a positive Voges-Proskauer reaction in contrast to the dog strains which were usually negative (31 per cent).

Methyl Red Reaction:

The methyl red reaction was studied in the same glucose phosphate broth medium that was used for the V.P. test. Only one strain, which produced only beta toxin out of 116 strains examined failed to give a positive or weak positive reaction.

The production of Indole:

Indole production was investigated in peptone water cultures after one and four days' incubation at 37°C. The same cultures were examined for evidence of motility. None of the 111 strains produced detectable amounts of indole and all were non motile.

Reduction of Methylene Blue:

The ability of staphylococci to reduce Methylene Blue was studied in a 1 per cent Methylene Blue milk solution.

Only 69 strains were examined and, with one exception, all reduced Methylene Blue milk, usually within two days. Visible clotting of the milk occurred with 52 strains.

Activity in Litmus Milk:

The inoculated media were incubated for ten days at 37°C. being examined at daily intervals for the presence of acid formation, coagulation and reduction of the litmus. Altogether 240 strains were examined of which 205 strains produced variable amounts of acid. A milk clot was formed by 178 strains and litmus milk reduced by 191 strains. There appeared to be no significant differences between dog and other animal staphylococci although strains from healthy dogs were less active in litmus milk than strains from infected ears.

TABLE 108

Animal staphylococci in litmus milk

Source of Strains	Number of Strains	Presence of Acid	Clot	Reduction
Dogs - infected ears	97	89	85	90
Healthy dogs - ears	84	64	50	52
nares	17	14	12	14
tonsils	11	7	4	5
Other dogs - lesions	8	8	7	7
Bovine lesions	11	11	9	11
Other animal lesions	12	12	11	12
	240	205	178	191

Urea hydrolysis:

Christensen's (1946) method was preferred to that of Stuart, Von Stratum and Rustigan (1945) because it did not give rise to false reactions due to the production of alkali from other constituents in the medium.

Altogether 247 strains from all sources were examined.

TABLE 109

Hydrolysis of Urea by animal staphylococci

Source of Strains	Number of Strains	Urea Hydrolysed (+)	(-)
Dogs - infected ears	58	57	1
Healthy dogs - ears	84	63	21
- nares	17	15	2
- tonsils	11	7	4
Other dogs - lesions	12	12	0
Bovine lesions	50	49	1
Other animal lesions	15	14	1
	247	217	30

Although there was no positive correlation between urea hydrolysis and coagulase production, the majority of strains that failed to split urea were coagulase negative.

Growth on MacConkey's bile-salt medium:

The growth characteristics of staphylococci on MacConkey's medium were used in a further attempt to differentiate canine and other animal staphylococci.

Overnight nutrient broth cultures were streaked on MacConkey plates and incubated at 37°C for 48 hours. The colour of the colonies were described as Dark Red, Pink (for shades between

between Bright Red to Salmon Pink) and Orange.

In the accompanying Table of results the symbols -, +, and ++ are used to describe no growth, a slight but obvious growth and an abundant growth.

TABLE 110

Growth characteristics of staphylococci on MacConkey's bile-salt medium

Source of Strains	Number of Strains	Degree of Growth -	+	++	Colour of Growth Red	Pink	Orange
Coagulase Positive Strains							
Dogs - infected ears	120	16	84	20	86	9	9
Dogs - other lesions	10	0	8	2	8	2	0
Bovine lesions	15	0	0	15	0	2	13
Other animal lesions	11	1	1	9	2	3	5
	156	17	93	46	96	16	27
Coagulase Negative Strains							
Dogs - infected ears	24	1	11	21	16	15	1
Dogs - other lesions	1	1	0	0	0	0	0
Bovine lesions	2	0	1	1	2	0	0
Other animal lesions	1	0	0	1	0	0	1
	28	2	12	23	18	15	2

Of the coagulase positive dog strains, 83 per cent failed to grow well on MacConkey's bile salt medium, whereas 92 per cent of other-animal "pathogens" produced an abundant growth. Most (72 per cent) of the former produced a dark red pigment which was shown by only 8 per cent of the latter. The orange type of pigment appeared to be characteristic of pathogenic other-animal strains, 69 per cent being of this type, as against only 17 per cent of dog staphylococci. It is also interesting to notice that 62 per cent of coagulase negative dog staphylococci grew abundantly, a figure which is comparable with that of the pathogenic animal strains which also grew very well on MacConkey plates, although the non-pathogenic dog strains rarely produced orange pigment.

PART VI.d. - SUMMARY:

Staphylococci from infected ears were examined and the findings compared with those from healthy dogs, from other lesions in dogs and from lesions in other animals.

They were all catalase positive, aerobic, Gram-positive cocci that grew well at 37°C and produced characteristic "paint-drop" colonies on the surface of solid media. Few of the coagulase positive dog strains formed the aureus pigment which is a feature of human and other-animal pathogenic staphylococci; and, of the few (18 per cent) that grew well on MacConkey's bile salt medium, most (72 per cent) formed small dark red colonies, in contrast to the abundant orange coloured growth of pathogenic strains from other animals. Although 84 per cent of canine strains actively hydrolysed the casein of milk agar media, only 35 per cent of the other-animal staphylococci did so, but weakly. Mannitol was fermented by 58 per cent of the pathogenic dog strains, usually after 2 or 3 days, whereas most (96 per cent) of the other-animal strains did so within 24 hours. There was, apparently, little difference between the staphylococci from different sources and their reactions to the nitrate, indole, urea, litmus milk and methyl red tests, except that acetylmethyl-carbinol was produced by only 31 per cent of the canine strains compared with 69 per cent from other animals. Nor was the ability to liquefy gelatin related to pathogenicity, as all coagulase positive strains, and the dog strains in particular,

particular, were actively proteolytic, as were 44 per cent of the coagulase negative strains.

As coagulase production is often used to indicate pathogenicity, the fact that 78 and 85 per cent respectively of the staphylococci from infected ears, and from other lesions in dogs, coagulated rabbit plasma, suggested that pathogenic strains were as common in external otitis as in other infectious conditions. Rabbit plasma was preferred to plasmas from other animals as it was coagulated by 85 per cent of the pathogenic dog strains compared with only 11 per cent with human plasma. On the other hand, 96 and 92 per cent of the pathogenic staphylococci from other animals coagulated both rabbit and human plasmas, respectively, within an hour. The rapid coagulation of human plasma may be associated with the production of alpha-toxin which is a feature of most human and animal pathogenic staphylococci, as similar strains from dogs which rarely formed this toxin, promptly coagulated rabbit but not human plasma. Rabbit plasma was also preferred, as it was coagulated by all delta-haemolysin producing strains, whereas human plasma was promptly coagulated only by strains that produced alpha-toxin, with or without delta-haemolysin. Conversely, although neither haemolysin was correlated absolutely with coagulase production, it appeared that, with dog strains, the presence of delta haemolysin was the more reliable index of pathogenicity.

These results may also suggest that the coagulase of dog

dog staphylococci is, in itself, distinct from the coagulase of other-animal staphylococci.

Alpha-toxin was produced by 73 per cent of pathogenic animal strains, whereas typical alpha-toxin, or an unidentified haemolysin which it closely resembled, was formed by only 34 per cent of the coagulase positive dog strains. Although Elek and Levy (1950) found that three distinct haemolysins (alpha, beta and delta) were associated with coagulase positive strains, whereas none was produced by coagulase negative strains, 33 of the 108 coagulase negative dog strains formed only beta-haemolysin as did one of the 10 strains from other animals. This, together with the fact that beta-lysin was produced by 89 and 82 per cent respectively of the coagulase positive staphylococci from dogs and other animals, confirmed the findings of Elek and Levy that the presence of beta-haemolysin is characteristic of animal staphylococci, although unrelated to pathogenicity. Although strains that produced delta-haemolysin, in the absence of alpha-toxin, were non-lethal to white mice, this did not impair its reliability as an index of pathogenicity as 93 per cent of coagulase positive strains produced this haemolysin compared with only 2 per cent of coagulase negative strains. The commonest of the seven possible combinations of haemolysins were, for coagulase positive canine strains, beta-delta (65 per cent), alpha-beta-delta (20 per cent) and alpha-delta (10 per cent); while for pathogenic strains from other animals they were alpha-beta-delta (45 per cent), beta-delta

beta-delta (27 per cent) and alpha-delta (18 per cent).

Although the properties of the individual haemolysins were similar to those of human staphylococci, the beta-toxin of canine strains often exerted a slight haemolytic effect on rabbit blood, while the synergistic haemolytic effect, on sheep red cells, of combinations of beta and delta haemolysins was not obtained on rabbit blood.

An unidentified haemolysin was described that produced zones of partial haemolysis on sheep and rabbit blood plates, was incompletely neutralised by commercial Staphylococcus alpha-antitoxin, was non-lethal to mice, was distinct from alpha, beta and delta haemolysins, and was produced only by a few dog strains, all of which were coagulase positive.

d) STREPTOCOCCI

Although the majority of haemolytic streptococci are pathogens, their haemolytic properties serve only as a broad differentiation of pathogenic and non-pathogenic strains irrespective of the identity of the individual species. As a result, many workers have attempted to differentiate the streptococci by fermentation reactions among whom Andrews and Horder (1906) defined a number of groups by name, viz: graminis, mitis, pyogenes, salivarius, and faecalis. This method is unsatisfactory, however, because of the variability of the reactions obtained from any one strain and so other tests were evolved. These included bile solubility, the reduction of methylene blue milk, heat resistance, the hydrolysis of æsculin and sodium hippurate, the final pH in glucose broth cultures, and the agglutination techniques of Griffiths (1926). All of these were overshadowed by the work of Lancefield (1925, 1933) who demonstrated the presence of both group and type specific antigens which could be readily identified by precipitation tests.

Dogs are frequently affected with streptococcal infections which vary from the peracute diseases of puppies to subacute and more chronic manifestations in older dogs.

The predominance of streptococci in acute tonsillitis in dogs was noted by Pilot, Buck, Davis and Eastman (1938), while Lancefield and Hare (1935) reported on the isolation of a Group G. haemolytic streptococcus from a case of otitis media. Little

Little additional information was forthcoming until Hare and Fry (1938a, 1938b), showed that haemolytic streptococci were responsible for anoestrus, abortion, adenitis, generalised septicaemias and a fatal infection of suckling puppies. They also recovered streptococci from throat and vaginal swabs, urine and cases of dermatitis. Of their 29 strains, 28 belonged to Group G, the remaining strain being in Group A.

Stableforth (1938) noted the presence of haemolytic streptococci in infected interdigital cysts and in cases of mastitis in the bitch, most of the strains falling into Group G, a few into Group C and one strain into Group B. This predominance of Group G. streptococci in canine infections was also observed by Minett and Ellis (1940), and by Garside (1947) who found that 82 per cent of haemolytic strains from infectious processes in dogs were in Group G.

Definition:

The streptococci were identified as Gram positive spherical or ovoid cells, occurring singly, in pairs or in chains but never in packets. They were invariably non-sporing, non-motile catalase negative aerobes which grew but slightly on artificial media in the absence of added blood, serum or glucose. Most species fermented a number of carbohydrates with the production of acid but not gas.

Classification:

A property which is much used in the classification of the streptococci is the ability of some strains to produce characteristic changes in media containing blood. Although Brown (1919) has described four types of haemolytic colony on 5 per cent horse blood agar plates, the streptococci in this present work will, for convenience, be described either as haemolytic or non haemolytic streptococci. The latter term will include all strains which do not give true beta haemolysis on horse blood agar.

All the haemolytic and a few of the non haemolytic strains will be divided into a number of different serological types by precipitin reactions carried out with type-specific anti-sera and suitably prepared bacterial extracts. As it was not possible to differentiate the majority of the non haemolytic species by these methods, they will be classified according to their biochemical reactions.

Cultural characters:

In glucose broth cultures at 37°C. most of the haemolytic strains produced a floccular or granular type of growth which settled out during overnight incubation leaving the supernatant clear, although an occasional strain (e.g. Group M) produced a degree of turbidity which was more characteristic of the non haemolytic varieties. The floccular type of growth was invariably associated with the long-chain forms in contrast to the moderate turbidity of the cultures of short-chain streptococci. The

The changes produced on blood agar and the colonial forms will be described during the discussion of the various types of organisms.

Haemolytic streptococci:

It will be recalled that the incidence of haemolytic streptococci in healthy dogs and in the external ears of dogs suffering from otitis was as follows:-

TABLE 111

The incidence of haemolytic streptococci in dogs

	Infected External ears	HEALTHY DOGS External ears	Middle ears	Nose	Tonsils
Number examined	523	70	50	35	35
Number positive	95	2	3	4	12
% Positive	18%	3%	6%	11%	34%

As only 21 of the normal dog swabs yielded haemolytic streptococci, this number was increased by including a number of strains from various normal tissues which differed biochemically from the majority of the strains on a given primary culture plate. The 95 "otitic strains" were compared with the 34 strains from healthy tissues. In order to assess the significance of these findings, it was decided to carry out a survey of haemolytic streptococci from infective material, other than "otitic swabs", which were examined in the Routine Diagnostic Laboratory of this

this School over a three-year period. Because of the large number of strains involved (190) they were examined only by the precipitin test, the results of which are included in the following Table.

TABLE 112

The distribution of Lancefield's Groups in haemolytic streptococci of canine origin

Source of Strains	Number of Strains Examined	GROUP (percent.) G.	L.	C.	D.	B.	M.	A.	H.
Healthy tissues	34	50	6	12	6	3	24	-	-
Otitis	95	59	18	16	4	1	2	-	-
Other infections	190	56	17	21	5	0.5	0.5	-	-

It will be seen from Table 112 that the incidence figures for the various Lancefield's Groups of haemolytic streptococci from cases of external otitis and other infectious processes in dogs are very similar. The descending order of frequency in both being Groups G. L. and C. D. M. and B. On the other hand, a comparison of the distribution of haemolytic streptococci in healthy and sick dogs shows that the carrier rate of Groups G. L. and C. is lower in healthy dogs, and that Group M. strains are especially common in the tonsils.

Group G. Haemolytic streptococci:

Various authors are of the opinion that Group G. strains are the commonest, and most important, pathogenic species in canine infections. (Hare and Fry, 1938; Garside, 1947; Laughton, 1948; and Davies, 1954). This was confirmed in this present work by the fact that 59 and 56 per cent respectively of haemolytic streptococci from otitis and other infections was placed in Group G. Although these figures are lower than those of Garside (1947), Gustaffson (1954) and Laughton (1948), namely 82, 77 and 70 per cent respectively, they compare very favourably with that of 61 per cent by Hare and Fry (1938), 60 per cent by Thal and Moberg (1956), and 50 per cent by Davies and Skulski (1956). In contrast to the infected animals the figure of 50 per cent, for Group G. strains from healthy dogs, is misleadingly high as three of the 34 strains were from the tonsil swabs of the same animal and were included in this present survey because it was found that they differed from each other in a number of minor biochemical features. Allowing for this, the incidence of Group G. strains in normal dogs was 45 per cent which is very similar to Shetty's (1949) figure of 46 per cent.

While it is obvious, from these findings, that Group G. strains are the most important pathogenic species in the dog, the carrier rate of 46 per cent is probably higher than is generally appreciated.

Groups L. and C.

Group C. haemolytic streptococci are common pathogens of domestic animals and a number of strains have been recovered from human infections. Group L. species, on the other hand, have only occasionally been recovered from the human throat but are fairly common pathogens of dogs and pigs. Apart from Garside (1947) who found that very few of his strains fell into Groups L. or C., the findings of the other authors, referred to above, are in general agreement with the results given in Table 112.

Group M.

Little was known of the incidence of Group M. strains in canine tissues until Laughton (1948) showed them to be the commonest species in the tonsils of healthy dogs, and that they accounted for 14 per cent of beta haemolytic strains in canine streptococcal infections. A somewhat lower figure was given by Shetty (1949) who found that Group M. strains accounted for 30 per cent of haemolytic streptococci in the naso-pharynx of normal dogs, which compares favourably with our figure of 24 per cent.

While only 2 per cent of the strains from cases of otitis were placed in this Group, which is very similar to the 2 per cent of Garside (1947), other workers have found that they are becoming increasingly numerous in canine infections. Davies and Skulski (1956) found that of 22 haemolytic strains from 'fading' puppies, 27 per cent were in Group M. Nevertheless, it is generally

generally agreed that Group M. strains, unlike those in Group G., are rarely of pathogenic importance.

Biochemical Reactions:

A number of subsidiary tests were used in conjunction with the serological investigations namely:-

(a) Sugar fermentation
(b) The hydrolysis of Sodium hippurate
(c) The reduction of Methylene Blue in milk
(d) The activity in Litmus milk media
(e) The activity in gelatin media
(f) The final pH in glucose broth
(g) Heat resistance at 60°C. for 30 minutes
(h) The ability to grow in the presence of bile salts (MacConkey's medium)

Fermentation reactions:

Fermentation reactions played a large part in the early attempts to classify the streptococci but they cannot now be accepted as an adequate means of distinguishing between the different groups and types. Nevertheless, fermentation and allied tests are of value as a means of supplementing the information already obtained by the study of their haemolytic ability and antigenic structure.

Each strain was tested for its ability to produce acid but not gas in arabinose, maltose, lactose, saccharose, trehalose, raffinose, inulin, glycerol, mannitol, sorbitol and salicin. The fermentation of aesculin in a bile containing medium was also included. It has already been shown (Page 87) that members of Groups G. L. C. and D., fell into a number of characteristic "fermentative patterns", the results of which were summarised in Table 35, page 86. It will now be convenient to consider the

TABLE 113.

Biochemical reactions of haemolytic streptococci.

Group G. strains. (pH. 4.5 - 4.95).

Number and source of strains.		Sodium hippurate	Aesculin	Arabinose	Maltose	Sucfose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus milk	Gelatin	
Normals	Otitis																G	L
0	1	+	+	+	+	+	+	+	-	-	+	-	-	+	-	AC-	+	-
1	1	+	+	-	+	+	+	-	-	-	-	-	-	+	-	AC-	+	-
0	2	-	+	-	-	+	+	-	-	-	-	-	-	+	-	ACR	+	-
0	10	-	+	v	+	+	+	-	-	-	-	-	-	+	-	Avv	+	-
2	3	-	+	v	+	+	+	-	-	-	+	-	-	+	v	ACR	+	-
0	11	-	+	v	+	+	+	-	-	-	-	-	-	+	-	AC-	v	-
0	1	-	+	v	+	+	+	-	+	-	-	-	-	+	-	Avv	v	-
0	5	-	v	v	+	+	+	+	-	-	+	-	-	+	v	ACR	v	-
6	10	-	+	v	+	+	+	+	-	-	v	v	-	+	v	ACv	+	-
1	3	-	+	-	+	+	+	+	+	-	-	-	-	+	-	Avv	+	-
0	5	-	+	-	+	+	+	v	-	-	-	-	-	-	-	AC-	+	-
0	4	-	+	-	+	+	-	v	-	-	-	-	-	v	-	ACR	v	-
4	0	-	+	v	+	+	+	v	-	-	-	+	-	+	-	ACR	+	+
2	0	-	+	v	+	+	+	v	-	-	-	+	-	+	-	ACR	+	-
1	0	-	+	-	+	+	+	+	+	-	-	-	-	+	-	ACR	+	+
17	56																	

Legend:

A = Acid
C = Coagulum formed
R = Litmus reduced

(+) = Reaction positive
(-) = Reaction negative
(v) = Reaction variable

G = Growth present in gelatin
L = Gelatin medium liquefied

TABLE 113.

Biochemical reactions of haemolytic streptococci.

Group G. strains. (pH. 4.5 - 4.95).

Number and source of strains.		Sodium hippurate	Aesculin	Arabinose	Maltose	Sucfose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus milk	Gelatin		MacConkey
Normals	Otitis																G	L	
0	1	+	+	+	+	+	+	+	-	-	+	-	-	+	-	AC-	+	-	-
1	1	+	+	-	+	+	+	-	-	-	-	-	-	+	-	AC-	+	-	-
0	2	-	+	-	-	+	+	-	-	-	-	-	-	+	-	ACR	+	-	-
0	10	-	+	v	+	+	+	-	-	-	-	-	-	+	-	Avv	+	-	-
2	3	-	+	v	+	+	+	-	-	-	+	-	-	+	v	ACR	+	-	-
0	11	-	+	v	+	+	+	-	-	-	-	-	-	+	-	AC-	v	-	-
0	1	-	+	v	+	+	+	-	+	-	-	-	-	+	-	Avv	v	-	-
0	5	-	v	v	+	+	+	+	-	-	+	-	-	+	v	ACR	v	-	-
6	10	-	+	v	+	+	+	+	-	-	v	v	-	+	v	ACv	+	-	-
1	3	-	+	-	+	+	+	+	+	-	-	-	-	+	-	Avv	+	-	-
0	5	-	+	-	+	+	+	v	-	-	-	-	-	-	-	AC-	+	-	-
0	4	-	+	-	+	+	-	v	-	-	-	-	-	v	-	ACR	v	-	-
4	0	-	+	v	+	+	+	v	-	-	-	+	-	+	-	ACR	+	+	-
2	0	-	+	v	+	+	+	v	-	-	-	+	-	+	-	ACR	+	-	-
1	0	-	+	-	+	+	+	+	+	-	-	-	-	+	-	ACR	+	+	-
17	56																		

Legend: A = Acid
C = Coagulum formed
R = Litmus reduced

(+) = Reaction positive
(-) = Reaction negative
(v) = Reaction variable

G = Growth present in gelatin
L = Gelatin medium liquefied

TABLE 114.

Biochemical reactions of haemolytic streptococci.

Group C. strains (pH. 4.6 - 5.3).

Number and source of strains		Sodium hippurate	Aesculin	Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus milk	Gelatin		MacConkey
Normals	Otitis																G	L	
0	1	+.	+	-	+	+	+	-	-	-	-	-	+	+	-	---	-	-	-
0	2	-	+	-	+	+	+	+	-	-	-	-	-	-	v	vvv	v	-	-
0	1	-	-	-	+	+	+	+	-	-	-	-	-	-	-	---	-	-	-
0	1	-	+	-	+	+	+	+	-	-	+	-	-	-	-	AC-	-	-	-
0	6	-	+	-	+	+	+	-	-	-	-	-	v	-	-	vvv	v	-	-
1	1	-	+	-	+	+	+	-	-	-	-	-	-	+	-	-CR	+	-	-
0	1	-	+	-	+	+	+	+	-	-	-	-	-	+	-	--R	+	-	-
0	1	-	+	-	-	+	+	+	-	-	+	-	-	-	-	---	-	-	-
2	1	-	+	-	+	+	-	v	-	-	-	-	v	-	-	A--	-	-	-
1	0	-	+	-	+	+	+	+	+	+	-	-	-	-	-	---	-	-	-
4	15																		

TABLE 115.

Biochemical reactions of haemolytic streptococci.

Group L. strains (pH. 4.8 - 5.1).

Number and source of strains		Sodium hippurate	Aesculin	Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus milk	Gelatin		MacConkey
Normals	Otitis																G	L	
0	3	-	+	-	+	+	-	+	-	-	-	-	-	-	-	Avv	+	-	-
0	2	-	+	-	+	+	-	+	-	-	-	-	-	+	-	Avv	+	-	-
0	2	-	+	-	+	+	-	+	-	-	+	-	-	-	-	Avv	+	-	-
0	6	-	-	-	+	+	+	+	-	-	-	-	-	-	-	Avv	+	-	-
0	2	-	-	-	+	+	+	+	-	-	-	-	-	+	-	Avv	+	-	-
2	2	-	+	-	+	+	+	+	-	-	-	-	-	+	-	Avv	+	-	-
2	17																		

TABLE 116a.

Biochemical reactions of haemolytic streptococci.

Group B. strains (pH. 4.4 - 4.6).

Number and source of strains		Sodium hippurate	Aesculin	Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus milk	Gelatin		MacConkey
Normals	Otitis																G	L	
1	2	+	-	-	+	+	+	+	-	-	+	-	-	+	-	ACv	+	-	-

TABLE 116b.

Biochemical reactions of haemolytic streptococci.

Group D. strains (pH. 4.2).

Number and source of strains		Sodium hippurate	Aesculin	Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus milk	Gelatin		MacConkey
Normals	Otitis																G	L	
2	4	-	+	-	+	+	+	+	v	-	+	+	+	+	+	ACR	+	+	+

TABLE 117.

Biochemical reactions of haemolytic streptococci.

Group M. strains (pH. 4.6 - 5.9).

Number and source of strains		Sodium hippurate	Aesculin	Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus milk	Gelatin		MacConkey
Normals	Otitis																G	L	
0	2	-	-	-	+	-	+	v	-	-	+	-	-	-	-	A--	-	-	-
2	0	-	-	-	+	+	-	-	-	-	-	-	-	v	-	v--	-	-	-
4	0	-	v	-	+	+	+	-	-	-	-	-	-	-	-	A--	-	-	-
1	0	-	-	-	+	+	+	-	+	-	-	-	-	-	-	A--	-	-	-

The results in Table 113 show that haemolytic streptococci give the following characteristic biochemical reactions:-

Group G.

All Group G. strains gave a very constant final pH. in 3-day old glucose broth cultures, the range being between pH. 4.5 to 4.95 with an average mean of pH.4.8. Only 2 strains hydrolysed sodium hippurate and another 2 strains failed to ferment aesculin within 48 hours. Lactose and salicin were usually promptly fermented, while all strains fermented saccharose and, apart from 2 strains, all fermented maltose. Only 41 per cent of strains attacked trehalose which is in contrast to Laughton's (1948) findings that 67 per cent were trehalose positive. The same author found that 71 per cent of her strains fermented sorbitol whereas neither our results nor those of Gustaffson (1955) showed sorbitol to be fermented by haemolytic Group G. streptococci from cases of canine otitis. It should also be noted that Laughton (1948) found that 67 per cent of her Group G. strains hydrolysed sodium hippurate whereas our results, and those of Gustaffson (1955) and Shetty (1949), show that the great majority of strains give negative reactions in hippurate broth.

It is generally agreed, however, that Group G. streptococci of canine origin do not usually ferment raffinose.

Group L.

All workers are agreed that Group L. strains do not hydrolyse

hydrolyse sodium hippurate and that the final pH. in glucose broth is somewhere in the range of 4.8 to 5.1. Unlike the Group G. strains, those of Group L. always fermented trehalose, maltose, saccharose and usually lactose but raffinose, salicin and glycerol were only rarely attacked. Although the formation of a coagulum in Litmus Milk media is not a feature of this Group of organisms (Garside, 1947), 9 of our 17 otitis cultures gave rise to a soft clot which developed slowly.

Group C.

The Group C. strains showed a wider pH range than other species, being between pH 4.6 and 5.3. Seven of the 15 Group C. strains were exceptional in that they fermented trehalose, although most strains resembled those of Group L. by their weak activities in salicin. They differed from Group L. strains, however, in that they grew poorly if at all on gelatin media at room temperature. The action of Group C. strains in Litmus Milk media was very variable and only one strain reduced Methylene Blue in milk.

Group M.

Group M. haemolytic streptococci were readily distinguished from other species on the surface of 5 per cent horse blood agar plates by the formation of very small, pinhead colonies which were invariably surrounded by a wide zone of true beta haemolysis. The ratio of the diameter of the haemolytic zone to the size of

of the colony was frequently of the order of 10 or 12 to 1. The Group M strains were further characterised by the fact that the growth was relatively poor and that the strains tended to die out rapidly.

Most strains fermented lactose, maltose and saccharose but only an occasional strain fermented salicin and glycerol. The fact that only one of the Group M. strains fermented trehalose agrees with Topley and Wilson (1955), Garside (1947) and Shetty (1949) but not with Laughton (1948) who reported that only 3 of her 28 strains failed to act on trehalose. The final pH ranged from 4.6 to 5.9, with an average mean of approximately 5.0. None of the strains showed visible growth on gelatin media at room temperature, sodium hippurate was not hydrolysed and only an occasional strain fermented aesculin.

Group B.

Only 3 Group B. strains were available for study, 2 of which were from the same dog. The final pH ranged from 4.4 to 4.6 and all strains hydrolysed sodium hippurate but failed to ferment aesculin. Lactose, maltose, saccharose, trehalose, glycerol and salicin were fermented but not arabinose, raffinose, inulin, mannitol or sorbitol. They all formed acid and coagulum in Litmus Milk, failed to reduce Methylene Blue in milk and grew in gelatin, without liquefying it, at room temperature. When tested with a known pathogenic strain of Str. agalactiae against a beta haemolysin producing staphylococcus on 3 per cent washed sheep

sheep blood agar plates, all 3 Group B strains showed a synergistic action which was indistinguishable from that produced by the Str. agalactiae strain. (Christie, Atkins and Munch-Petersen, 1944).

Group D.

Only six haemolytic Group D. strains were tested, 2 from healthy dogs and 4 from the ears of dogs suffering from otitis. They all grew well on MacConkey's bile salt medium, resisted 60°C. for 30 minutes, grew well on gelatin and liquefied it when incubated at room temperature, reduced Methylene Blue and showed acid, clot and reduction in litmus milk media. They were much more active in sugars than were the other species of haemolytic streptococci, only arabinose and inulin being unaffected by most strains. Their final pH in glucose broth was consistently low (pH 4.2) and none of the strains hydrolysed hippurates.

Litmus Milk Media:

The activities of the haemolytic streptococci in Litmus Milk media merits further discussion as it appeared that members of different Lancefield's Groups could be distinguished by their ability, or otherwise, to form a coagulum in this medium. The results of this investigation are summarised in Table 118.

TABLE 118

The activities of haemolytic streptococci in Litmus Milk media

Effects produced in Litmus Milk			Group and number of strains reacting in the manner indicated					
Acid	Clot	Reduction	G.	L.	C.	D.	B.	M.
+	+	+	39	3	3	5	2	0
+	+	-	29	8	7	0	1	0
+	-	-	1	5	1	0	0	7
-	-	-	2	0	4	0	0	2
-	-	+	0	0	4	0	0	0
+	-	+	2	3	0	0	0	0
			73	19	19	5	3	9

These results indicate that haemolytic strains from dogs give the following characteristic reactions in Litmus Milk media. Group M strains do not form a coagulum, do not reduce the litmus and produce little or no acid, whereas strains of Groups B. and D. always form a firm clot, produce acid and usually reduce the litmus. While there is no hard and fast division between members of the other groups, the great majority of Group G. strains produce acid and a coagulum, whereas this latter property is a very variable feature of strains of Groups L. and C. Clot formation by Group L. strains is generally delayed and weak and,

and, in contrast to Group C. strains, they usually produce visible acid change in the indicator medium.

The "C.A.M.P." Test:

Although only a proportion of Group B. strains are beta haemolytic, Christie, Atkins and Munch-Petersen have shown that all strains of Str. agalactiae can lyse sheep or ox cells in blood agar in the presence of a beta haemolysin-producing staphylococcus. Although our 3 Group B. strains from dogs produced zones of complete haemolysis on horse blood agar plates, the effect on sheep cells was enhanced in the presence of staphylococcal beta haemolysin. This was demonstrated by streaking an overnight culture of a staphylococcus, which produced only beta haemolysin, on the surface of an agar plate containing thrice-washed sheep cells in the proportion of 5 per cent. The streptococcal strains under investigation were streaked, in parallel, at right angles to the staphylococcus and incubated at 37°C. for 24 hours in an atmosphere of air plus 20 per cent CO_2. The margins of the haemolytic zones surrounding the culture 'streaks' were examined in the region of the staphylococcal haemolysin for evidence of synergism or increased lysis, in the case of incomplete haemolytic strains. Of the total of 75 strains tested, 45 produced beta haemolysis on horse blood agar plates, 10 produced only a partial or alpha type of haemolysis and 20 strains had no effect on horse cells. The results of this investigation are summarised in Table 119.

TABLE 119

The haemolytic activities of streptococci on sheep cells, in the presence of staphylococcal beta haemolysin

Type of haemolysis on horse blood agar	Number of Strains	Lancefield's Group	Increased haemolytic effect on sheep cells in the presence of staphylococcal beta haemolysin				
			++++	+++	++	+	-
	2	B	2	-	-	-	-
	20	G	1	3	3	2	11
Complete	10	L	-	-	-	2	8
	10	C	-	-	1	-	9
	3	M	-	-	-	-	3
Partial	10	-	-	-	1	4	5
Not haemolytic	20	-	-	-	-	3	17
	75		3	3	5	11	53

Only 3 of the 75 strains gave an increased haemolytic effect on sheep cells comparable with that of the known strain of Str. agalactiae. Two of these were the Group B dog strains and the other was a typical Group C. strain. Altogether 7 of the 20 Group C. strains had a synergistic haemolytic effect on sheep cells in the presence of staphylococcal beta haemolysin, a property which was manifest but to a lesser degree by one Group C strain

strain and an alpha haemolytic strain. Although this alpha haemolytic strain was re-examined serologically, it did not fall into any of the known Lancefield's Groups.

NON HAEMOLYTIC STREPTOCOCCI:

As the non haemolytic streptococci could not be differentiated serologically, each strain was classified by its physiological and biochemical characters. The reactions studied were the hydrolysis of sodium hippurate, the fermentation of sugars including aesculin, the action on methylene blue milk and litmus milk media, the production of ammonia in peptone water, heat resistance, and the ability to grow in and liquefy gelatin. Of the 170 strains which were examined in detail, 91 were from different tissues of clinically healthy dogs and 79 were from the external ears of dogs suffering from otitis. The results of these investigations are summarised in Table 120.

TABLE 120

The biochemical characters of non-haemolytic streptococci.

Positive reactions to :-	Source and number of strains giving the reactions indicated. Healthy dogs. External ears	Middle ears	Nose	Tonsils	Rectum	Dogs with External otitis	Total
Sodium hippurate	-	-	1	1	3	22	27
Aesculin	23	9	16	18	19	75	160
Arabinose	14	1	9	7	13	38	82
Maltose	25	10	16	20	20	79	170
Saccharose	21	6	12	17	19	76	151
Lactose	23	7	14	18	20	67	149
Trehalose	21	4	16	13	13	65	132
Raffinose	5	1	2	7	6	23	44
Inulin	-	1	1	6	-	4	12
Glycerol	11	-	11	8	9	34	73
Mannitol	15	2	9	11	10	50	97
Sorbitol	15	1	7	9	6	47	85
Salicin	19	2	11	13	20	77	142
Methylene blue	17	-	9	4	17	63	110
Litmus milk -							
Acid	20	5	14	17	20	75	151
Clot	17	4	14	13	20	47	115
Reduction	24	4	13	14	20	59	134
Growth in bile salt	19	-	5	4	15	58	101
Gelatin liquefied	-	-	1	2	4	27	34
NH_3 from peptone water	13	-	5	4	13	13+	
Number of strains: examined.	25	10	16	20	20	79	170

Note: (+) = Ammonia was produced by 13 of the 29 alpha haemolytic strains.

These results show that most of the non haemolytic streptococci fermented all of the sugars except inulin and raffinose. Strains from healthy and infected external ears differed from each other in that the former did not liquefy gelatin or hydrolyse hippurates and were much less active in salicin. On the other hand, the non haemolytic varieties from the middle ears of healthy animals were exceptional in that their fermentative powers were weak, and they did not reduce methylene blue in milk, grow in bile salt media, liquefy gelatin or produce ammonia in peptone water cultures. Although the sugar reactions of the strains from the nose, tonsil and rectal swabs were very similar, the rectal strains were generally more active in milk media. They also grew better in the presence of bile salts and usually formed ammonia in peptone water.

It will be recalled that non haemolytic streptococci were recovered from 25 per cent of infected ears and from 30, 14, 46 and 51 per cent respectively of the external and middle ears, the anterior nares, and the tonsils of healthy dogs.

As a result of their biochemical reactions, details of which are summarised in Table 121, it was possible to subdivide the 170 selected non haemolytic strains into nine main groups

TABLE 121.

Species distribution within the non-haemolytic group of streptococci.

Source	Strept. faecalis	Strept. liquefaciens	Strept. bovis	Strept. equinus	Strept. salivarius	Strept. mitis	Strept. glycerinae	Lancefield's Group C.	Lancefield's Group L.	Total
	Number of strains in each group.									
External otitis	26	27	11	5	6	-	1	1	2	79
Healthy dogs										
External ears	13	-	3	2	5	2	-	-	-	25
Middle ears	-	-	2	2	5	1	-	-	-	10
Nose	6	1	2	2	5	-	-	-	-	16
Tonsils	2	2	4	2	8	2	-	-	-	20
Rectum	9	4	1	-	5	1	-	-	-	20
Total:	56	34	23	13	34	6	1	1	2	170

There is little reference in the literature to the incidence and distribution of non haemolytic streptococci in infectious conditions in dogs, and it is, therefore, of interest that three species were predominant in external otitis, viz. Str. faecalis, Str. faecalis var liquefaciens and Str. bovis. While two of these were equally common to both healthy and infected external ears, the third species Str. faecalis var liquefaciens occurred only in the otitic material. It will also be seen that these three species accounted for 70 per cent of the rectal strains. On the other hand, the commonest non haemolytic varieties from the middle ears and tonsils of healthy dogs were members of the Str. salivarius-mitis group.

It will now be convenient to discuss the biochemical characters of the non haemolytic species in some detail.

The Str. faecalis and Str. liquefaciens group:

Although it is generally accepted that neither of these species is of pathogenic significance, they have been described as the causative organisms of a number of human infections. (Topley and Wilson, 1955). Str. faecalis was first described by Andrewes and Horder (1906) as the predominant streptococcus of the human intestine and is also to be found in the alimentary tract of animals (Stableforth, 1946). Altogether 56 faecalis strains were identified, 26 of which were from otitic material and 13, 6, 2 and 9 were from the external ears, nose, tonsils and rectum respectively, of healthy dogs. The frequency and the distribution of different types within this group are shown in Table 122.

TABLE 122.

Biochemical reactions of non-haemolytic streptococci.

A. Strept. faecalis (56 strains).

pH. 4.0 - 4.6

Number of strains	Sodium hippurate	Aesculin	Arabinose	Maltose	Saccharose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus Milk. A.	Litmus Milk. C.	Litmus Milk. R.	Growth in bile salts	Gelatin G.	Gelatin L.	Ammonia in peptone water
2	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	-	-
2	+	+	+	+	-	+	+	-	-	-	v	v	+	+	+	+	+	+	+	-	+
4	+	+	v	+	+	+	+	-	-	v	+	v	v	+	+	v	+	+	+	-	+
4	-	+	v	+	+	+	+	+	-	-	+	v	+	+	+	+	+	+	+	-	+
8	-	+	v	+	+	+	+	-	-	-	+	v	+	+	+	+	+	+	+	-	+
15	-	+	v	+	+	+	+	-	-	+	+	+	+	+	v	v	+	+	+	-	+
4	-	+	v	+	-	+	+	-	-	v	+	+	+	+	+	v	+	+	+	-	+
2	-	+	v	+	+	-	+	-	-	-	v	+	+	+	+	-	v	+	+	-	+
5	-	+	v	+	+	+	+	+	-	-	-	-	+	+	v	v	+	+	+	-	+
1	-	+	-	+	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+	-	+
4	-	v	v	+	+	+	+	+	+	v	v	-	+	v	+	v	v	+	+	-	+
2	-	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+
1	-	+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	+
1	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	+
1	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+

Note:
A = Acid formed in litmus milk
C = Clot formed in litmus milk
R = Litmus reduced
G = Growth present in gelatin
L = Gelatin liquefied
v = Variable reaction

The results in Table 122 show that the great majority of faecalis strains of canine origin split aesculin but rarely hydrolysed sodium hippurate. Although their sugar reactions were variable, most strains formed acid but not gas from maltose, saccharose, lactose, trehalose, salicin and mannitol. Methylene blue in milk was usually reduced, while in litmus milk most strains showed acid, clot and reduction. With only 2 exceptions, all strains grew in the presence of bile salts, and formed ammonia in peptone water. They all grew on gelatin media, without liquefying it, at room temperature.

Str. faecalis var liquefaciens was distinguished from Str. faecalis by the fact that most strains liquefied gelatin media promptly. Apart from the action in sodium hippurate broth, which was hydrolysed by 19 of the 34 liquefaciens strains, the biochemical reactions of these two species were very similar (Tables 122 and 123). Of 34 strains of Str. liquefaciens, 27 were recovered from otitic material, 1 from a nasal swab, 2 from the tonsils and 4 were from rectal swabs of healthy dogs.

Str. bovis:

Str. bovis was first described by Winslow and Palmer (1910) as the prevailing streptococcus in the ox and, while Fuller and Armstrong (1913) considered it to be identical with Str. salivarius of the human throat, Orla-Jensen (1919) suggested that it might be a new species on account of its fermentative activities.

TABLE 123. & TABLE 124.

Biochemical reactions of non-haemolytic streptococci.

B. Strept. faecalis var liquefaciens (34 strains)

pH. 4.0 - 4.3

Number of strains	Sodium hippurate	Aesculin	Arabinose	Maltose	Saccharose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus Milk A.	Litmus Milk C.	Litmus Milk R.	Growth in bile salts	Gelatin G.	Gelatin L.	Ammonia in peptone water
3	-	+	v	+	+	-	+	-	-	v	+	+	+	+	+	+	+	+	+	+	+
3	v	+	v	+	-	+	+	-	-	+	+	+	+	+	v	+	+	+	+	+	+
8	-	+	-	+	+	+	+	-	-	+	+	+	+	+	+	v	+	+	+	+	+
9	+	+	v	+	+	+	+	-	-	+	+	+	+	+	+	v	+	+	+	+	+
3	v	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
5	+	+	v	+	+	+	+	+	-	v	v	v	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
1	-	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
1	-	+	-	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+

C. Strept. bovis (23 strains)

pH. 4.0 - 4.8

1	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	-	+	+	-	-
3	-	+	v	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	+	-	-
1	-	+	+	+	+	+	+	-	+	-	+	-	-	+	+	+	-	-	+	-	-
6	-	+	+	+	+	+	-	+	-	-	-	v	+	v	+	v	v	v	+	-	-
4	-	+	+	+	+	v	+	-	-	+	-	-	+	+	+	+	+	+	+	-	-
2	-	+	-	+	+	+	-	v	v	-	-	-	+	-	+	v	v	-	+	-	-
3	-	+	v	+	-	+	+	-	-	v	+	+	v	-	v	v	v	-	+	-	-
2	-	+	+	+	+	+	v	-	-	-	+	-	+	v	+	-	-	-	+	-	-
1	-	+	-	+	+	-	+	-	-	-	+	+	+	+	+	-	-	+	+	-	-

Note:

A = Acid formed in litmus milk
C = Clot formed in litmus milk
R = Litmus reduced
G = Growth present in gelatine
L = Gelatin liquefied
v = Variable reaction

rectal swab, and two each were from the external and middle ears, the nose and tonsils.

TABLE 125

Biochemical reactions of non haemolytic streptococci

D. Str. equinus (14 strains)

pH 4.2 - 4.6

Number of Strains	Aesculin	Arabinose	Maltose	Saccharose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene Blue	Litmus Milk A.	Litmus Milk C.	Litmus Milk R.	Gelatin G.	Gelatin L.
5	+	-	+	+	-	v	v	-	-	-	v	+	v	+	-	-	+	-
2	+	v	+	+	-	+	+	-	-	+	-	-	-	+	-	+	-	-
6	+	v	+	-	-	-	-	-	v	-	-	-	v	v	v	v	v	-
1	+	-	+	+	-	+	-	-	+	-	-	-	-	+	+	-	+	-

NOTE: None of the strains of Str. equinus grew in the presence of bile salts, produced ammonia in peptone water, or hydrolysed sodium hippurate.

None of the equinus strains formed ammonia in peptone water, grew in the presence of bile salts or liquefied gelatin at room temperature. Only an occasional strain reduced methylene blue in milk while in litmus milk media, although acid was usually formed, only a few strains formed a clot and reduced the indicator. Unlike the other species described above, Str. equinus frequently failed to ferment salicin, sorbitol, mannitol, raffinose, arabinose and trehalose, but lactose and inulin were never attacked.

Although aesculin was promptly split, only an occasional strain hydrolysed sodium hippurate.

Str. salivarius and Str. mitis:

Andrewes and Horder (1906) described Str. salivarius and Str. mitis as the predominant species in the human throat and suggested that, in the absence of a clear-cut division between the two, the mitis type was possibly a variant of Str. salivarius. These findings were largely supported by later workers (Topley and Wilson, 1955), until Sherman et al (1941) claimed to be able to differentiate them by a few physiological characters.

In this work, 40 strains were identified as members of the salivarius-mitis group of which only 6 were from otitic material. Of the remaining 34 strains which were isolated from healthy dogs, 7 were from external ears, 6 from middle ears, 5 from the nose, 10 from the tonsils and 6 were from the rectum.

Although the number of mitis strains is unfortunately small, it is interesting to notice that, unlike Str. salivarius, none of them split aesculin, or fermented glycerol, mannitol or sorbitol. While the results in Tables 126 and 127 would appear to indicate that the salivarius and mitis strains were almost as active in litmus milk media as Str. faecalis and Str. liquefaciens, it is emphasised that, although acid was usually produced, the formation of a coagulum and the reduction of the indicator was not marked.

TABLE 126 & TABLE 127

Biochemical reactions of non-haemolytic streptococci.

E. Strept. salivarius (34 strains)

pH. 4.0 - 4.4

Number of strains	Sodium hippurate	Aesculin	Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Methylene blue	Litmus Milk A.	Litmus Milk C.	Litmus Milk R.	Growth in bile salts	Gelatin G	Gelatin L	Ammonia in peptone water
2	-	+	v	+	+	+	+	v	-	+	-	+	+	-	v	-	-	v	+	-	-
2	-	-	v	+	+	+	+	+	-	-	+	-	+	-	+	-	-	+	+	-	-
1	-	+	-	+	+	+	+	+	-	-	-	-	-	+	+	-	-	+	+	-	-
3	-	+	-	+	+	+	+	+	+	-	+	+	+	v	+	+	+	-	+	-	-
2	-	+	v	+	+	+	v	+	-	-	-	-	+	-	+	+	+	-	+	-	-
8	-	+	v	+	+	+	+	-	-	v	v	-	v	v	+	+	+	-	+	-	-
1	-	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	-	-
5	-	+	v	+	+	+	-	-	-	v	-	-	+	-	+	v	+	-	+	-	-
7	-	+	-	+	+	+	-	-	-	-	-	-	v	-	v	v	v	-	+	-	-
2	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
1	-	+	-	+	-	+	+	-	-	-	-	+	+	+	+	+	+	-	+	-	-

F. Strept. mitis (6 strains)

pH. 4.2 - 4.3

1	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	+	-	-
1	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
2	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
2	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	v	v	-	+	-	-

Note:

A = Acid formed in litmus milk
C = Clot formed in litmus milk
R = Litmus reduced

G = Growth present in gelatin
L = Liquefaction of gelatin
v = Variable reaction

Str. glycerinae:

The single strain of Str. glycerinae fermented all of the sugars except inulin, split aesculin, hydrolysed sodium hippurate, produced ammonia in peptone water, grew in the presence of bile salts, reduced methylene blue milk and gave acid, clot and reduction in litmus milk. Although growth occurred in gelatin, there was no evidence of liquefaction.

PART VI.e. - SUMMARY:

The streptococci were said to be haemolytic or non haemolytic depending on their ability to haemolyse 5 per cent horse blood agar.

Haemolytic streptococci:

Several workers, including Hare and Fry (1938), Garside (1947) and Laughton (1948) consider Group G. strains to be the most important pathogenic species in streptococcal infections in dogs. In this present work, not only was this opinion confirmed by a study of a number of haemolytic strains from various infected tissues but it was also shown that Group G. streptococci were as common and, presumably, as pathogenic in the infected acoustic meatus. In contrast to the strains of Groups L and C which accounted for 18 and 16 per cent respectively of the haemolytic streptococci in otitic material, the Group M strains were rarely found in infected ears (0.5 per cent) but were isolated from no fewer than 24 per cent of healthy dogs.

The biochemical activities of a number of strains in each group were also investigated and, while these were insufficient to differentiate members of Groups G. L. and C., several features appeared to be more characteristic of one species than another. Group G. strains usually fermented salicin, frequently saccharose, but not sorbitol; most Group C. strains attacked saccharose but rarely salicin and sorbitol: and Group L. strains, while

while fermenting saccharose but not mannitol, gave variable reactions in salicin. In milk media methylene blue was reduced by some of the Group G. and Group C. strains but not by Group L. strains. In litmus milk media only members of Groups G. and L. consistently produced acid, while clot formation and the reduction of the indicator was a feature of Group G. strains but not of Groups L. and C.

Sodium hippurate was hydrolysed by the three Group B. strains, by an occasional strain in Groups L. and C., but not by the other haemolytic streptococci.

The final pH in three-day-old glucose broth cultures (tested with a Marconi pH meter Type T.F. 889/1) placed the strains of Groups G. C. L. and M. in the "low-acid" group, and those of Groups B. and D. in the "high-acid" group.

A study of the haemolytic activities of a number of strains, in the presence of staphylococcal beta toxin showed an increased haemolytic effect on sheep red cells by both of the Group B. strains and most of the Group G. strains. A similar effect was not produced by other haemolytic strains nor by the non haemolytic varieties.

Non haemolytic streptococci:

All the non haemolytic streptococci, apart from one Group C and two Group L. strains, were classified by their biochemical reactions into the following species, viz., Str. faecalis,

Str. faecalis, Str. liquefaciens, Str. bovis, Str. salivarius, Str. mitis and Str. glycerinae. Of these, the faecalis-liquefaciens group accounted for 67 per cent of the non haemolytic strains in infected ears, whereas only 8 per cent were in the salivarius-mitis group. In healthy dogs, however, faecalis species were commoner than salivarius and mitis strains in the external ears (52 and 28 per cent respectively) and in the rectal swabs (65 and 30 per cent respectively); but the latter varieties predominated in the middle ears (60 and 0 per cent respectively) and in the tonsils (50 and 20 per cent respectively). The incidence of Str. faecalis and Str. liquefaciens in the external ears of both healthy and affected dogs suggests that their presence is the result of faecal contamination, whereas the predominance of the salivarius-mitis group in the middle ears and tonsils would indicate that they are normal inhabitants of these tissues. As the latter group of organisms were rarely isolated from infected ears, and as the "faecal" strains were frequently present in the external ears of healthy dogs, it is unlikely that the non haemolytic group, as a whole, are of pathogenic significance in external otitis.

PART VI.

f). Pityrosporum.

The sub-family Cryptoccoideae includes the genus Pityrosporum of which the type species is Pityrosporum ovale. These are oval or flask-shaped asporogenous, non-mycelial yeasts, without fermentative ability.

Rivolta (1873) was probably the first to describe these organisms which he observed in a case of psoriasis in man, calling them Cryptococcus psoriasis. In the following year a more detailed description was given by Mallassez (1874) of sporing fungi, as distinct from mycelium forming fungi, in cases of pityriasis simplex. These were later described by Sabouraud (1904) as bottle shaped or budding yeast cells, the bud being separated from the mother cell by a narrow cross wall.

While Ciferri and Redaelli (1929) referred to slight fermentative activity, Lodder (1934) considered that fermentation was absent and that cultivation of these yeasts on malt agar was very difficult.

According to Lodder and van Rij (1952) the genus Pityrosporum includes the two species P. ovale and P. pachydermatis, the latter of which has been described inadequately, whereas Gordon (1951) has described a third species from skin lesions in man which he calls P. orbiculare.

Schoop (1951) in a preliminary report, considers that external otitis in dogs may be a blastomycosis, as many of the ears which he examined showed a profusion of blastomycetes, to the exclusion of bacteria. Although he also showed that they were also present in healthy ears, he is of the opinion that their presence is of aetiological significance in ear canker in dogs.

Gustafson (1954), also in a preliminary report, found "asporous yeasts" in approximately 65 per cent of affected canine ears and claims that he was able to transmit the infection in healthy dogs. He further considers that, with two exceptions, they were all members of the genus Pityrosporum. Similar yeasts were also recovered from a number of healthy ears.

It will be recalled, from the results in Parts I and II of this thesis, that Pityrosporum species were recovered from 36 per cent and 44 per cent respectively of normal and infected ears, and that there was some doubt as to their aetiological significance.

Cultural characters.

In a number of cases heavy pure growths were obtained in primary cultures on Sabouraud's maltose agar plates without the addition of oleic acid, olive oil or other fatty substances. (Plate 2). On this medium, after incubation at 37°C. for 48 hours, small heaped colonies developed which reached full size (2.0 - 2.5) in from 4 to 5 days, their colour being invariably off-white or ivory. A slower but satisfactory amount of growth was obtained on the same medium at 27°C. After 6 days at 37°C. the colonies became darker, to buff or 'old ivory', while the

the surface resembled frosted glass and acquired a dimpled appearance that was not unlike the skin of an orange. (Plates 52 and 53). On maltose agar slopes the growth became drier and more wrinkled with age, until (after 6 to 9 months at 17°C.) it was of a dark brown or chocolate brown colour. Although the organisms grew on primary culture on the surface of 10 per cent horse blood agar, there was no evidence of haemolysis and the colonies remained minute, in spite of prolonged incubation. Subcultivation on this medium was generally unsuccessful. On nutrient agar and inspissated bovine serum slopes the degree of growth of subcultures of Pityrosporum was scant or absent but on glycerine agar a fairly profuse growth was obtained. Glucose broth cultures were of interest in that, while turbidity and sedimentation were absent, a fine lace-like flaky growth was seen on the surface of the medium which settled to the foot of the tube with gentle agitation. The amount of growth rarely covered the entire surface of the medium, however, most strains showing growth over only a third to a half of the surface area. All strains grew well on MacConkey's bile salt medium when pale pink colonies, of approximately 1.5 mm. in diameter, were produced after 48 hours incubation at 37°C. As the pH. of the bile salt and horse blood agar media were approximately the same, this suggests that Pityrosporum species of canine origin do not only favour the presence of bile salts in the medium but that they are also capable of digesting and absorbing fats. This may account for their presence in situations such as the external acoustic meatus.

PLATES 52 and 53.

Pityrosporum species.

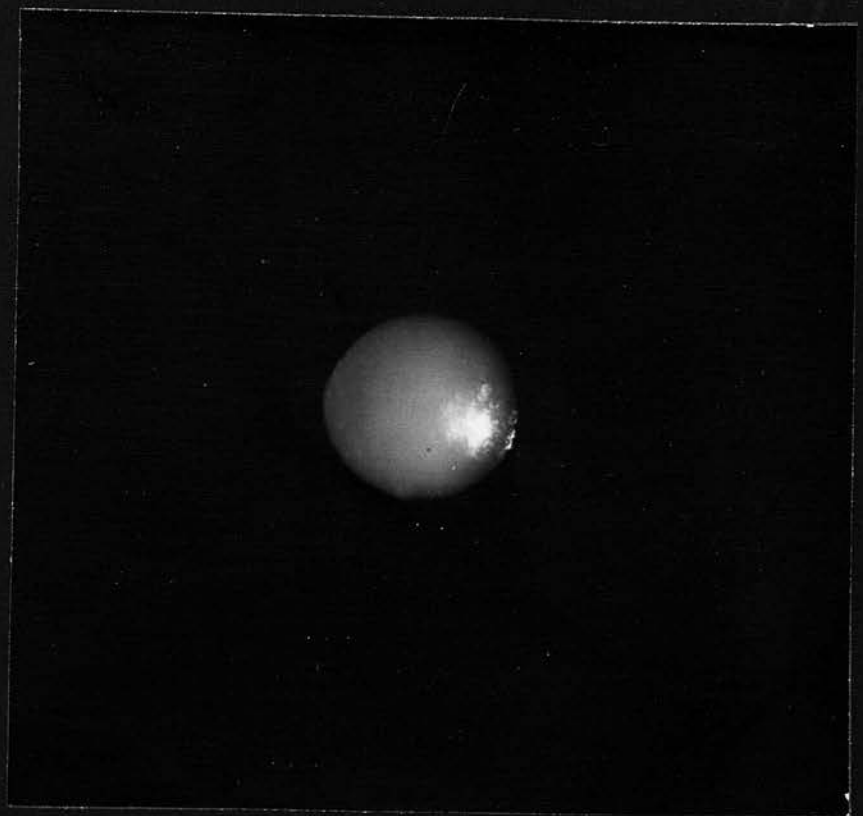
Typical surface colony of Pityrosporum species on a Sabouraud's maltose agar plate after 6 days at 37°C.

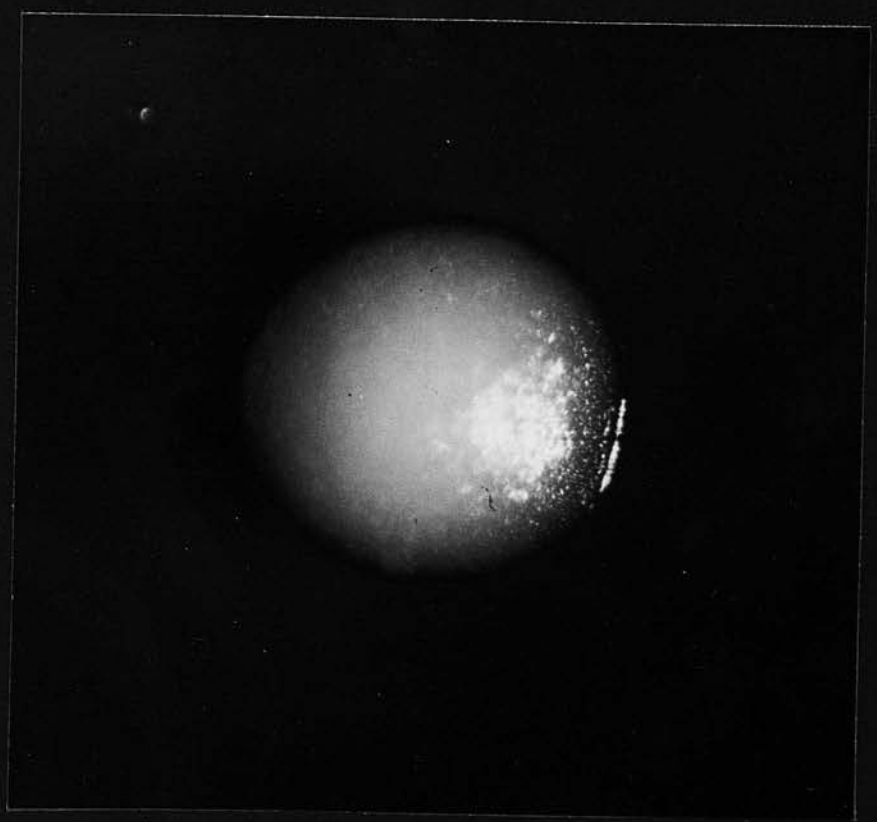
Plate 52. (Magnification x 12)

Showing the 'frosted glass' appearance of the surface of the colony.

Plate 53. (Magnification x 18)

Showing the dimpled or 'orange skin' appearance of the surface of colony.





A number of strains were grown on various egg media including Dorset's egg, Glycerol egg, Lowenstein-Jensen's and Finlayson's media. Little or no growth occurred on Glycerol egg and Lowenstein-Jensen's media although both Dorset's and Finlayson's media produced an abundant growth. That Finlayson's medium, which contains an extract of Mycobacterium phlei, was undoubtedly the medium of choice also points to the lipolytic activity of this group of organisms.

Both methylene blue and litmus milk media were unaffected by Pityrosporum species and, on milk agar, there was no evidence of casein hydrolysis although a reasonably heavy growth occurred within 2 days. Stab inoculation of gelatin resulted in a large circular golden-yellow disc-like growth on the surface of the medium but no growth occurred along the length of the stab, nor did liquefaction take place within 12 weeks. All strains produced traces of ammonia, within 7 days, when grown on Christensen's urea medium at 37°C. but none fermented 'sugars', either in peptone water or bile salt sugar media. None of the strains formed indole or produced hydrogen sulphide.

The optimum temperature for growth was 37°C. when a freshly prepared moist medium was preferred. No growth occurred anaerobically although all strains grew well in an atmosphere containing 20 per cent added CO_2.

A number of strains were subcultivated on maltose agar plates containing different concentrations of penicillin but in no case was there evidence of increased growth.

Sensitivities to antibiotics and antiseptics.

As the prevalence of asporogenous yeasts in otitic material suggested that they may be of aetiological significance, it was decided to treat a selected number of dogs with suitable antiseptic substances, to ascertain whether otitis persisted in the absence of these organisms. With this in mind a number of Pityrosporum strains were tested for their in vitro sensitivities to five common antibiotics and six different antiseptics. The results of this investigation showed that all strains were completely resistant to penicillin, streptomycin, chloromycetin, aureomycin and terramycin and that of the antiseptics tested, those with the greatest fungistatic action were Lugol's iodine, 'Roccal', 'Cetavlon', Hibitane', 'Penotrane' and proflavine, in that order. Affected ears, with the characteristic chocolate-brown discharges, that gave heavy pure cultures of Pityrosporum, were treated with at least one of the proprietary antiseptic preparations but, although sterile cultures were subsequently obtained, there was no obvious improvement in the clinical condition. These findings appear to confirm the earlier impression that the presence of Pityrosporum species in infected canine ears are not, by themselves, responsible for the condition. In other words, although the presence of Pityrosporum species may aggravate the condition, their removal will not, by itself, result in complete clinical recovery unless steps are then taken to promote rapid healing of the existing lesion in the external acoustic meatus.

Animal experiments.

Maltose agar cultures were applied to the shaved and scarified skin of white mice without producing visible effects within eight weeks. Intraperitoneal, intravenous and subcutaneous inoculations in mice, of saline suspensions of organisms, also failed to produce a reaction.

Morphology.

Films prepared from otitic material and stained by Gram's method showed positively staining oval or flask-shaped yeast bodies of approximately 2 - 3 x 4 - 5μ in size. (Plates 54 & 55). Culture smears were similar.

The majority of strains showed budding forms in which the blastospores were separated from the mother cells by narrow, unstained but well defined transverse septa. As well as being Gram positive, most of the adult forms showed numerous intercellular granules, while the older forms, and the very young daughter cells, were readily decolourised. Another feature of Pityrosporum was the presence of a thickened cell wall, or capsule. Some of these points are clearly visible in Plate 56. It was also noticed that in stained smears of older cultures, or of cultures grown on unsuitable media, small projections occurred on either side of the mother cells in the region of the transverse septum, suggesting the presence of scar tissue or of a protoplasmic collar of unknown function. Irrespective, however, of the medium used or the length of the incubation period, multiple budding or mycelial elements were never present.

PLATES 54 and 55.

Pityrosporum species.

Direct smears of otitic material stained by Gram's method.

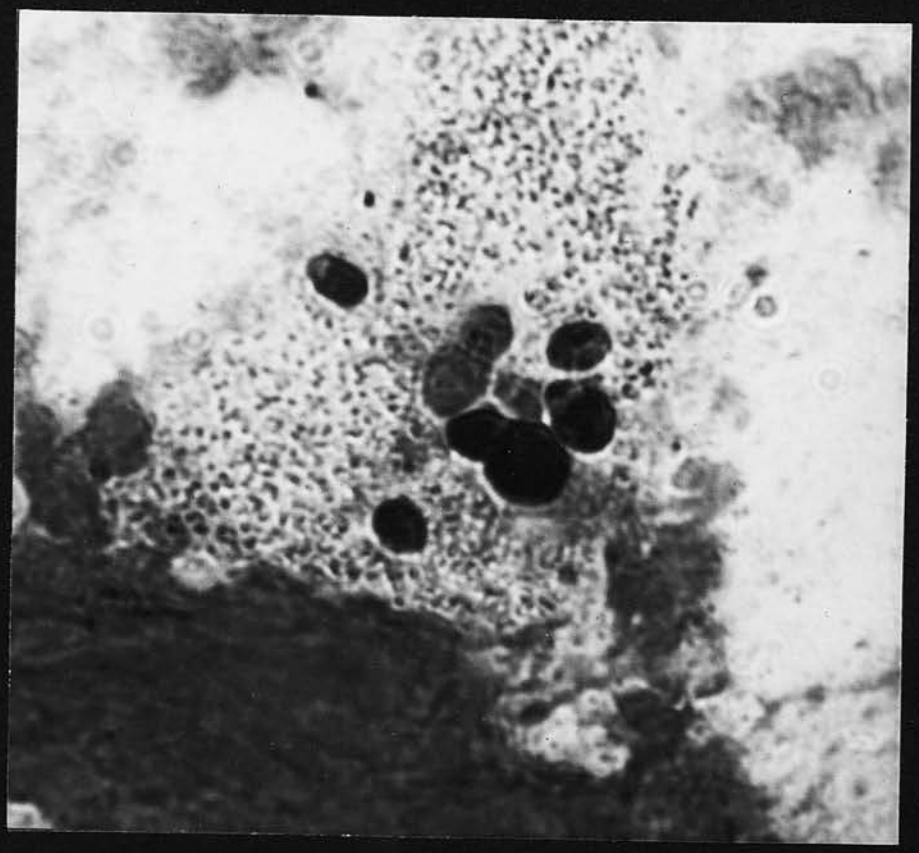
Plate 54 (Magnification x 2,200)

Showing both Gram positive and Gram negative yeasts (Pityrosporum) at different stages of sporulation. Notice the unstained transverse septum. The presence of a protoplasmic collar of scar tissue is suggsted by the yeast in the top left-hand corner.

Plate 55 (Magnification x 2200)

Showing a typical cluster of Gram positive, flask-shaped, budding yeasts.

The presence of a thick outer cell wall or capsule is suggested by many of the yeasts.



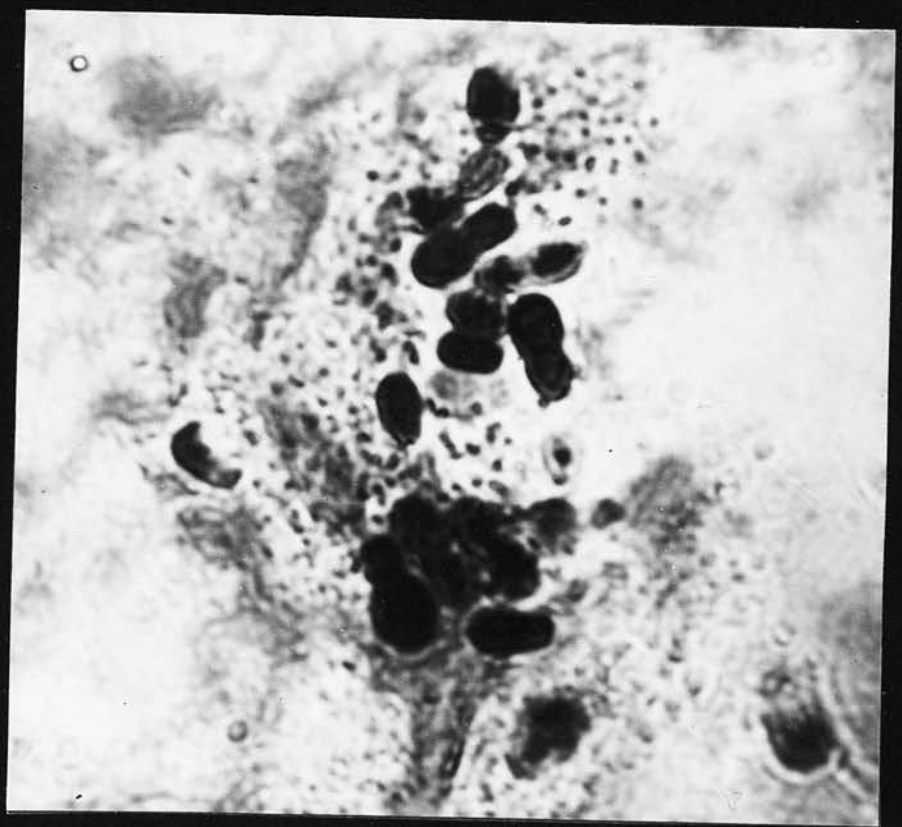


PLATE 56.

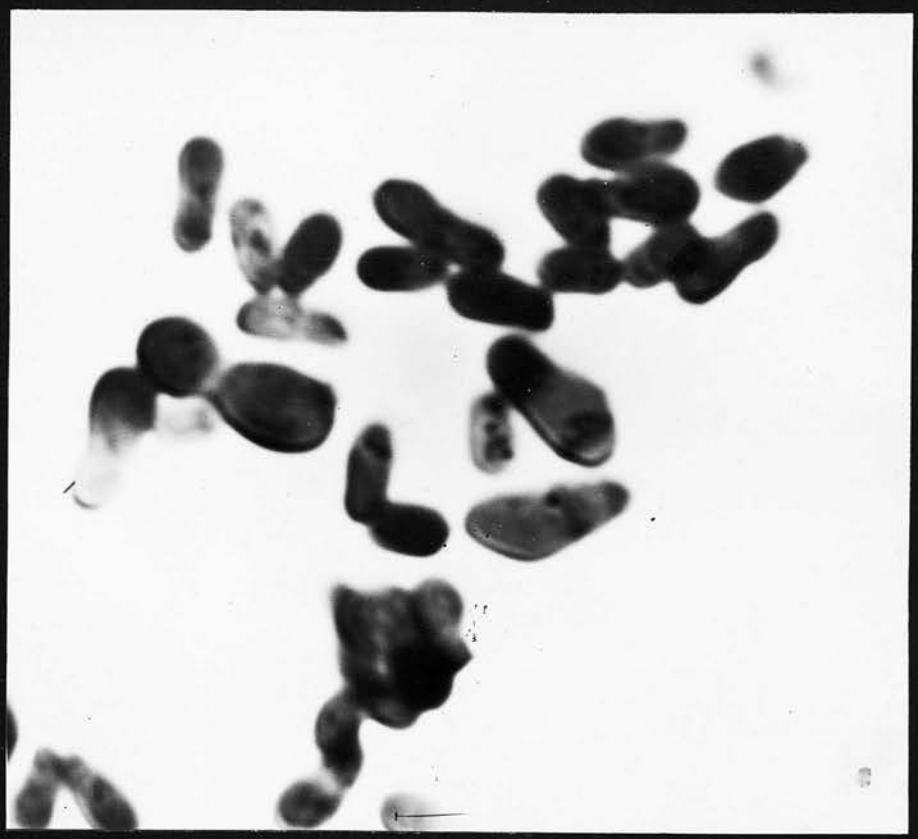
Pityrosporum species.

Smear prepared from a 48 hour culture, on maltose agar, of Pityrosporum species. Stained by Gram's method.

Showing Gram positive budding yeasts with intercellular granules, thickened cell walls and transverse (unstained) septa between the daughter blastospore and the mother cell.

Note that in one of the yeasts (left centre of plate) the blastospore is almost fully mature and is assuming the rounded appearance of the mother cell.

Magnification x 2400



Capsular staining.

In Plates 55 and 56, attention was drawn to the existence of a thick-walled structure surrounding the cell. By staining films firstly by Gram's method and then by adding a small quantity of Indian ink, which was spread carefully over the dried film, the the cytoplasm of the cell appeared as a deep blue colour surrounded by a clear space between this and the ink particles. Culture films stained by Muir's method showed a pale blue capsule surrounding a purplish red or carmine cytoplasm. The capsules were particularly prominent in the younger cells and around the developing blastospores. (Colour negative, Plate 61). The relationship of the capsule to the cell is also clearly shown in Plates 57 to 60. These films which were stained by Muir's method are duplicated, the second plate in each series being photographed, with suitable filtration, to differentiate the blue capsule from the carmine cytoplasm.

Reproduction.

Although Martin-Scott (1952) is of the opinion that single thick-walled spherical spores are an optical illusion, due to the bottle shaped yeast standing on its end, he agrees with the general opinion that P. ovale multiplies by simple budding. He further considers that the daughter cells are already flask-shaped and are never present as single cells before separation from the mother cell. As far as the canine strains from otitic material are concerned there is little doubt but that oval single-cell spores do exist as these were frequently observed both

PLATES 57 and 58.

Pityrosporum species.

Smears prepared from a 48 hour Sabouraud's maltose agar culture of Pityrosporum species and stained by Muir's method.

Plate 57 (Magnification x 2200)

Shows an accumulation of blue (?) capsular material around the developing blastospores.

Plate 58 (Magnification x 2200)

The same field as in Plate 57 but photographed with a yellow filter to obtain contrast. The presence of a capsule is suggested along one side of the lowest of the three yeast bodies.

PLATES 59 and 60.

Pityrosporum species.

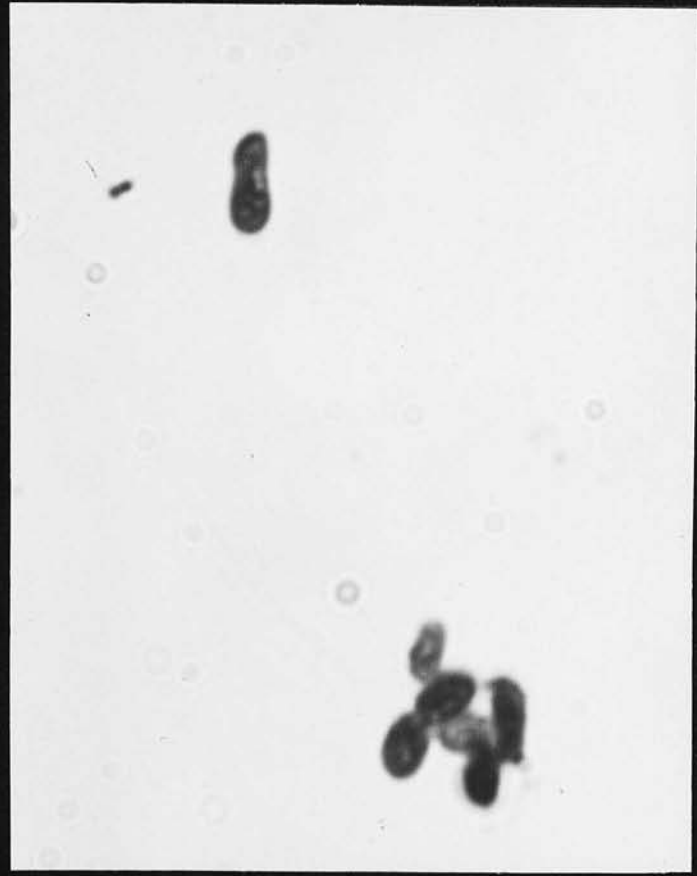
Smear prepared and stained as in Plates 57 and 58.

Plate 59 (Magnification x 2400)

Unfiltered photograph shows the faint outline of capsular material.

Plate 60 (Magnification x 2400)

The capsular material around each yeast body is clearly shown by the use of a yellow filter.



both in direct smears and in films prepared from cultures. It is considered most unlikely that these are flask-shaped cells standing on end.

The following cycle of events is suggested for canine strains of Pityrosporum:- The young, single, oval cells give rise to a tiny polar bud which slowly develops on a broad base within the the parent capsule. This blastospore has, at first, a rounded end and straight parallel sides but, in a very short time, this gives way to the more typical biconvex appearance. When it reaches half the size of the mother cell, the daughter cell or blastospore becomes separated from it by a thin, sharply demarcated transverse septum near which a collar-like projection is sometimes seen. This may be the remains of scar tissue from a previous cell-division. Occasionally the daughter cell becomes detached at this stage, as if on a hinge, and is to be seen lying at right angles to the mother cell, although it is realised that this stage may be due to mechanical injury during the preparation of the stained films. Failing this the blastospore continues to expand, becoming more spherical as it does so, until the two parts are approximately equal in size and are separated by a short neck on a very narrow base although still within the parent capsule. Separation may then occur spontaneously when the two oval cells are free to continue the process of multiplication.

In spite of the detailed examinations above, it was not possible to differentiate individual strains within the genus, either morphologically or biochemically. Nevertheless, it is of interest to compare their more important characters with those of the other three recognised species.

PLATE 61.

Pityrosporum species.

Colour transparency showing the blue capsular material surrounding the carmine cytoplasm of the yeast cell.

Magnification x 2400

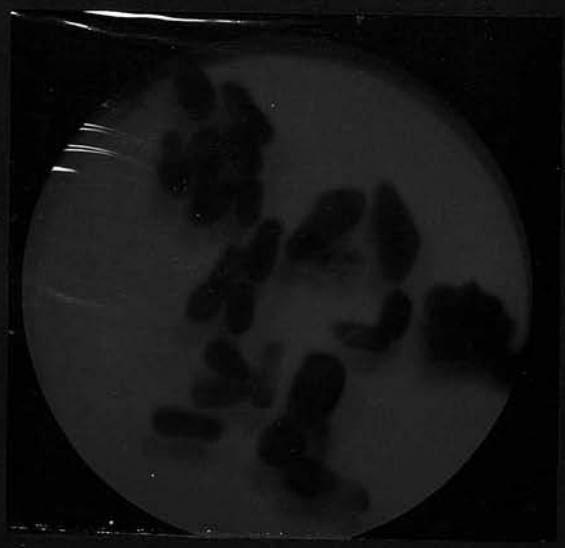


TABLE 128.

A comparison of the principal characters of the three recognised species in the genus Pityrosporum with those of canine strains.

Character	P. ovale	P. orbiculare	P. pachydermatis	Pityrosporum spp. (canine).
Author	Lodder et al. (1952).	Gordon (1951)	Weidman (1925)	This work.
Host	Man	Man	Rhinoceros	Dog
Commonest site.	Scalp and skin lesions.	Shoulder, upper trunk and arms.	Skin lesions.	External ear.
Cell shape	Oval	Spherical	Egg-shaped	Oval
Approx. size in μ.	2-3 x 4-6	2.1 - 4.8	1.5-3 x 2.5-5	2-3 x 4-8
Budding	Broad base	Narrow base	Broad base	Broad base
Growth on culture-media	Difficult	Difficult	Very difficult to keep alive.	Very easy
Substance necessary for growth.	Oleic acid	Olive oil and fats.	Not known, but some probably essential.	None required.
Colony type	2 mm. in diam. Buff colour. Domed.	As for P. ovale	Dark, cream to yellow. Doughy consistency. Smooth surface.	2-2.5 mm. in diam. Dry, frosted glass. Dimpled surface. Domed.

The results in Table 128 show that Pityrosporum species from the external ears of dogs are very different from other members of the genus, but whether it is desirable to assign to canine strains the specific name P. canis must be decided by competent mycologists, as a more detailed study of these organisms is required.

It is also of interest that, in their Survey of Animal Mycoses in Britain, Ainsworth and Austwick (1955) reported the isolation of P. pachydermatis Weidman which was associated with ulceration of the conjunctiva and ear tips of a dog. During this present work the writer observed Pityrosporum species in smears of otitic material from a Large White sow which differed from the canine strains by the fact that they were Gram-negative, pear-shaped yeasts that would not grow on the usual mycological media.

PART VI f. - Summary.

Pityrosporum species were isolated from 36 and 44 per cent respectively, of the healthy and infected external ears of dogs, and from no other site.

They all grew well on Sabouraud's maltose agar, both at 25°C. and 37°C., and on certain other media, without the addition of oleic acid. An abundant growth was obtained in the presence of bile salts and on egg media containing an extract of Mycobacterium phlei.

They were fully resistant, in vitro to penicillin, streptomycin, chloromycetin, aureomycin and terramycin but were sensitive to Logol's iodine, 'Roccal', 'Cetavlon', 'Hibitane', 'Penotrane', and proflavine, in that order.

Skin scarification and inoculation of suspensions of organisms, by various routes, failed to produce a reaction in white mice.

A thick-walled structure resembling a capsule surrounded the cell body, which was generally strongly Gram-positive.

Multiplication took place by the production of a single blastospore, on a broad base, without the formation of mycelia.

Pityrosporum species from dogs were distinct from P. ovale and P. orbiculare of man and P. pachydermatis of the rhinoceros.

Although the presence of Pityrosporum may, like dandruff of the scalp, give rise to irritation of the epithelium of the external acoustic meatus, their removal from the ears of a number of cases of otitis externa did not result in a marked clinical improvement of the condition.

PART VII.

The antibiotic sensitivities of the more important bacteria in canine external otitis.

The determination of the sensitivities of micro-organisms to antibiotics has now become one of the most important duties of the bacteriological laboratory.

In recent years a great deal of work has been published describing modifications of a number of in-vitro methods of which the diffusion, serial dilution and turbidometric techniques are most widely used. While some of the methods tend to give results of a qualitative rather than of a quantitative nature, their accurate interpretation depends upon a number of variable factors e.g. the size of the inoculum, the composition of the growth medium, the presence in the culture of resistant variants, the stability of the particular antibiotic, the accuracy with which the final dilutions are prepared, the length of incubation of the test, the rate of growth of the organism to be tested and the depth of the medium in the agar diffusion methods. Nevertheless, by standardising the conditions of the tests and by adhering rigidly to a definite procedure, many of these disadvantages are overcome and results are obtained that are sufficiently accurate to be of clinical value.

Methods:

A number of strains of the more frequently occurring bacteria in external otitis, namely staphylococci, streptococci, E. coli, Pseudomonas and Proteus were examined by disc diffusion and tube dilution techniques, for their in vitro sensitivities to penicillin, streptomycin, chloromycetin, aureomycin and terramycin. The disc diffusion techniques are increasingly used for determining antibiotic sensitivities as, apart from the saving in time and materials, they are more accurate than the "ditch" and "cylinder" methods and give closely similar results when carried out in parallel with serial dilution tube tests. (Gould and Bowie, 1952). The results of the disc diffusion tests were correlated with the size of the zones of inhibition by plotting the sensitivities of a selected number of strains, determined by tube tests, against the corresponding zones of inhibition with the plate test. By measuring the diameter of the zones of inhibition, the approximate Minimal Inhibiting Concentration (M.I.C.) was then obtained from the appropriate curve. (The Minimal Inhibiting Concentration, hereafter referred to as the M.I.C., was defined as the lowest concentration of antibiotic to which the organism was sensitive in vitro.)

Because of their growth characters on the surface of solid media, doubts arose as to the suitability of the disc diffusion methods for determining the antibiotic sensitivities of Proteus and Pseudomonas. In an attempt to overcome this secondary

secondary spreading type of growth in the zones of inhibition, MacConkey's medium was substituted for blood agar, but this method was later abandoned because of the variable results obtained due, it was thought, to the irregular rate of diffusion of some of the antibiotics in the presence of bile salts. The sensitivities of Proteus and Pseudomonas were, therfore, determined by serial dilution tube tests.

The disc diffusion techniques consisted of flooding the surface of a blood agar plate with a 1 in 10 dilution of an 18 hour broth culture, the plate being then dried in an incubator in the inverted position for 20 minutes when impregnated filter-paper discs were placed on the surface of the medium at suitably placed intervals. The plate was then incubated at 37°C. for 18 hours when the diameter of the zones of inhibition were measured in millimeters. Care was taken that the depth of the medium in each plate did not exceed 2 mm. The antibiotic solutions were prepared in distilled water in the following concentrations, 1 ml being added to the appropriate bottle containing 100 sterile discs :-

Penicillin	100 units/ml.
Streptomycin	1000 mcg. /ml.
Chloromycetin	2500 mcg. /ml.
Aureomycin	5000 mcg. /ml.
Terramycin	2500 mcg. /ml.

Although the serial tube dilution method is more laborious, it is probably the most accurate procedure for determining a

a strain's sensitivities to antibiotics. The method used was that of a two-fold serial dilution in broth, plus indicator, twelve tubes being used of which the last was a control. Each tube contained 0.5 ml of the dilution of the antibiotic plus 1.5 ml of a 1: 50 culture dilution. The M.I.C. was taken as the the amount of antibiotic in the last tube in which there was no visible growth after 24 hours at 37°C.

Results.

Staphylococci.

A great deal of work has been published regarding the antibiotic sensitivities of pathogenic staphylococci of human origin. Although Spink (1951) claimed that about 12 per cent were naturally resistant to penicillin and that of 104 strains, 5 were resistant to terramycin and 7 to chloromycetin, without the patients having been exposed previously to antibiotic therapy, the number of resistant forms had greatly increased in recent years. Linsell (1952) noted that of 118 strains of pathogenic human staphylococci 19 per cent were resistant to penicillin, 2 strains were resistant to terramycin and 3 strains showed a degree of resistance to aureomycin. On the other hand, Barber and Rozwadowska-Dowzenko (1948) have shown that in a London Hospital, 14 per cent were penicillin resistant in 1946, and that the incidence of resistant strains had increased in the next two years

years to 38 and 59 per cent respectively.

There are, unfortunately, very few references to the sensitivities of pathogenic staphylococci of canine origin although Farrag (1949) reported that of 25 strains of animal origin, all but one were sensitive to 0.5 units/ml of penicillin. Three years later, Dam (1952) showed that of 21 strains from cases of canine otitis 15 were sensitive to penicillin, 16 to streptomycin, 19 to terramycin and 20 to chloromycetin and aureomycin.

It is interesting to recall that Blair, Carr and Buchman (1946) studied the effect of penicillin on staphylococci and noted that, as resistance developed there was a reduction in pigment production, in the fermentation of mannitol and in the production of alpha toxin. The ability to coagulate rabbit plasms was not impaired, however.

In this thesis, 225 strains of coagulase positive staphylococci were examined of which 160 were from cases of canine otitis, 20 were from other lesions in dogs, and 45 were from mastitis and other infectious processes in cattle. It is emphasised that in the results which are summarised in Table 129, strains that required 2 units/ml., or more, to inhibit growth were held to be resistant, as sensitivities to higher concentrations of penicillin are probably of little therapeutic value.

TABLE 129.

The in vitro sensitivities of staphylococci to penicillin.

M.I.C. in units/ml.	Minimal Inhibiting Concentration — Number and source of strains & year of examination				
	Canine otitis			Other lesions in dogs	Bovine lesions
	1953	1954	1955	1956	1956
0.01	-	-	-	-	-
0.02	9	10	13	5	3
0.03	7	3	14	2	3
0.04	27	6	11	2	-
0.05	9	5	7	4	3
0.1	10	8	4	2	2
0.2	1	3	1	1	1
0.3	-	-	1	-	2
0.4	-	-	-	-	1
0.5	-	-	-	-	1
1.0	-	-	-	-	3
2.0 - R.	3	2	6	4	27
Number of strains examined.	66	37	57	20	45

Most of the otitis strains were sensitive to 0.05 units/ml. of penicillin and 40 per cent of the bovine strains were sensitive to 0.2 units/ml. In contrast to the canine strains, however, only 7 per cent of which were resistant, no fewer than 60 per cent

per cent of the bovine strains continued to grow in 2.0 units/ml of penicillin. This high incidence of bovine resistant strains was probably due to the fact that they were mostly isolated from clinical cases of mastitis in herds that were known to have been exposed to penicillin therapy. Although this suggested that many of the bovine strains had acquired a resistance to penicillin the low incidence of canine resistant strains was not unexpected as very few dogs had been treated previously with penicillin. It was also noticed that the few resistant dog strains resembled staphylococci from human sources as they formed typical aureus pigment and both alpha and delta haemolysins. This suggested that certain strains may occur in infected canine ears as a result of cross-infection from humans.

The M.I.C. of the other antibiotics was expressed in mcg./ml and strains that required at least 30 mcg./ml to prevent visible growth were held to be resistant.

A review of the literature suggests that the average M.I.C. of streptomycin, for human staphylococci, is in the region of 4.0 mcg/ml although the values quoted range from 0.078 - 0.3 mcg./ml (Hamburger and Muething, 1951) for very sensitive strains, to 0.5 - 16.0 mcg./ml (Garrod, 1948), 0.5 - 32.0 mcg. /ml (Linsell, 1952), 5.0 - 25.0 mcg./ml (May and Morley, 1952) and 0.5 - 62.6 mcg./ml (Spink, 1951), for the more resistant strains.

The sensitivities of animal staphylococci to streptomycin are summarised in Table 130.

TABLE 130.

The in vitro sensitivities of staphylococci to streptomycin.

Minimal Inhibiting Concentration.

Number and source of strains and year of examination.

M. I. C. in mcg./ml	Canine otitis			Other lesions in dogs	Bovine lesions
	1953	1954	1955	1956	1956
0.1	1	1	1	-	1
0.3	4	6	7	3	2
0.5	10	3	9	5	2
0.75	24	11	22	2	2
1.0	15	5	12	3	3
2.0	4	4	1	2	8
3.0	3	4	-	2	17
4.0	-	1	-	1	6
5.0	-	-	-	-	2
6.0	-	-	-	-	-
8.0	1	-	-	-	-
12.5	2	-	-	1	1
25.0	1	1	3	-	-
30 - R.	1	1	2	1	1
Number of strains.	66	37	57	20	45

These results show that most of the canine and bovine staphylococci were inhibited by 4.0 mcg./ml, the average M.I.C. being 1.8 and 2.6 mcg./ml respectively, while the number of streptomycin resistant strains is very much lower than with penicillin.

Some of the human strains are very sensitive to chloromycetin, values of 0.3 and 0.9 mcg./ml being given by Hamburger and Muething (1951) and Spink (1951), respectively. Other strains, however, are less sensitive, M.I.C's. of 10.0, 16.0 and 62.5 mcg./ml being quoted, respectively, by May and Morley (1952), Valentine and Shooter (1954) and Spink (1951).

TABLE 131.

The in vitro sensitivities of staphylococci to chloromycetin.

M.I.C. in mcg./ml.	Canine otitis			Other lesions in dogs	Bovine lesions
	1953	1954	1955	1956	1956
0.1	-	-	-	-	-
0.3	-	-	-	-	-
0.5	-	-	-	-	-
0.75	1	1	2	-	1
1.0	3	3	8	1	6
2.0	-	2	6	-	2
3.0	23	15	26	6	7
4.0	23	8	6	6	15
5.0	-	-	-	1	4
6.0	12	4	4	3	4
8.0	-	-	2	-	-
12.5	2	1	2	1	4
25.0	-	-	-	1	1
30 - R.	2	3	-	1	1
No. of strains.	66	37	57	20	45

The results in Table 132 show that chloromycetin was less effective than streptomycin against canine and bovine strains, as the average M.I.C. was 2.6 and 4.7 mcg./ml respectively, as against 1.8 and 2.6 mcg./ml. respectively, for streptomycin.

Perusal of the relevant literature shows that human staphylococci are very sensitive to both aureomycin and terramycin, the M.I.C's being about the same for both namely, 0.2 to 6.0 mcg./ml and 0.2 to 12.5 mcg/ml respectively, although less sensitive strains are not uncommon.

TABLE 132.

The in vitro sensitivities of staphylococci to aureomycin.

M.I.C. in mcg./ml.	Canine otitis			Other lesions in dogs	Bovine lesions
	1953	1954	1955	1956	1956
≤0.05 to 0.05	6	-	2		1
0.075	2	2	2		2
0.1	9	6	10		8
0.3	15	9	20		6
0.5	27	8	13	EXAMINED	14
0.75	-	8	5		7
1.0	4	4	1		2
2.0	3	-	2		1
3.0	-	-	2		1
4.0	-	-	-		-
5.0	-	-	-	NOT	-
8.0	-	-	-		2
12.5	-	-	-		-
30 - R.	-	-	-		1
No. of strains	66	37	57		45

TABLE 133.

The *in vitro* sensitivities of staphylococci to terramycin.

M.I.C. in mcg./ml.	Number and source of strains and date of examination. Canine otitis 1953	Canine otitis 1954	Canine otitis 1955	Other lesions in dogs 1956	Bovine lesions 1956
0.05	-	-	-	-	-
0.075	-	-	-	-	1
0.1	3	7	10	6	21
0.3	8	3	3	-	9
0.5	12	12	8	3	8
0.75	37	9	21	5	2
1.0	6	4	9	4	2
2.0	-	2	4	-	-
3.0	-	-	2	2	1
4.0	-	-	-	-	-
6.0	-	-	-	-	-
8.0	-	-	-	-	-
12.5	-	-	-	-	-
30 - R.	-	-	-	-	1
No. of strains.	66	37	57	20	45

The results in Tables 132 and 133 show that most of the animal staphylococci were inhibited by 0.05 - 0.75 mcg./ml. of Aureomycin and 0.1 - 2.0 mcg./ml of terramycin, 17 (8 per cent) of which were sensitive to 0.075 mcg./ml of the former compared with only a single (bovine) strain which was sensitive to the same concentration of the latter.

Streptococci.

May and Morley (1952) examined 57 strains of haemolytic streptococci and found that all were sensitive *in vitro* to 10.0 and 5.0 mcg./ml of chloromycetin and terramycin respectively and that most of the strains were inhibited by 0.25 units /ml and 25.0 mcg./ml of penicillin and streptomycin respectively. According to Valentine and Shooter (1954), the average M.I.C.'s (in mcg./ml) of penicillin, streptomycin, aureomycin, terramycin and chloromycetin, for pathogenic strains of *Str. pyogenes*, were 0.01, 50.0, 0.5, 0.5 and 3.0 respectively; the corresponding figures for faecal streptococci being 2.5, 50.0, 0.6, 0.3 and 10.0 respectively. The action of antibiotics on streptococci from cases of bovine mastitis was studied by Edwards (1952) who reported that in Hartley's broth growth of *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis* was inhibited by 0.04 mcg./ml of penicillin, by 1.0 - 20.0 mcg./ml of streptomycin and by 0.03 mcg./ml of aureomycin.

As the streptococci were thought to be the least important of the commoner bacterial species in cases of canine otitis (Part II), only 40 strains were studied half of which were haemolytic and half non-haemolytic.

The summarised results of this investigation are to be found in Table 134.

TABLE 134.

The in vitro sensitivities to five antibiotics of haemolytic and non-haemolytic streptococci. (20 strains of each).

Canine strains only.

M.I.C. in uu/ml	PEN.		M.I.C. in mcg/ml	STREP.		CHLOR.		AUREO.		TERRA.	
	Hy.	NHy.		Hy.	NHy.	Hy.	NHy.	Hy.	NHy.	Hy.	NHy.
2.0 >	-	-	> 30.0	3	2	-	-	-	-	-	-
2.0	-	-	30.0	-	2	-	2	-	-	-	-
1.0	-	2	25.0	-	5	-	-	-	-	-	-
0.5	-	6	20.0	-	5	-	1	-	-	-	1
0.4	-	-	15.0	-	3	-	3	-	-	-	-
0.3	-	11	12.0	3	3	-	-	-	1	-	-
0.2	-	1	9.0	-	-	2	12	-	3	-	9
0.1	-	-	6.0	11	-	6	2	-	12	-	7
0.05	1	-	3.0	3	-	2	-	5	4	5	3
0.03	8	-	1.0	-	-	6	-	4	-	10	-
0.01	11	-	0.5	-	-	4	-	11	-	5	-

PEN. = penicillin
STREP. = streptomycin
CHLOR. = chloromycetin
AUREO. = aureomycin
TERRA. = terramycin

Hy. = haemolytic streptococci.
NHy. = non-haemolytic streptococci.

The results in Table 134 show that only the haemolytic strains were sensitive to 0.5 units or less of penicillin, whereas the non-haemolytic streptococci required at least 40 times this amount to inhibit growth. Similar differences were observed with their sensitivities to other antibiotics.

Escherichia coli.

Weil and Stempel (1953) studied the in vitro activities of six antibiotics against 38 strains of "Colon Group" organisms. The most effective were chloromycetin, terramycin, aureomycin and streptomycin, in that order; all were resistant, however, to penicillin and erythromycin. A similar investigation was carried out by May and Morley (1952) who found that of 262 strains of coliform organisms none was inhibited by 0.25 units /ml of penicillin, 29 per cent were inhibited by 5.0 mcg./ml of streptomycin, 78 per cent by 10.0 mcg./ml of chloromycetin and 80 per cent by 5.0 mcg./ml of terramycin. Although Valentine and Shooter (1954) found that resistant strains were not uncommon, most E. coli strains were inhibited by 6.0, 5.0, 5.0 and 10.0 mcg./ml of streptomycin, aureomycin, terramycin and chloromycetin respectively.

The sensitivities of 40 strains of E. coli from cases of canine otitis were studied by the disc diffusion techniques, when the following results were obtained. (Table 135).

TABLE 135.

The *in vitro* sensitivities to five antibiotics of *E. coli* of canine origin.

Antibiotic	No. of strains examined	Minimal Inhibiting Concentration (mcg./ml.)						
		0.75	1.5	3.0	6.0	12.5	25.0	>25.0
Penicillin	40	-	-	-	-	-	-	40
Streptomycin	40	-	3	8	15	9	1	4
Chloromycetin	40	-	-	3	23	9	2	3
Aureomycin	15	-	-	1	4	6	4	-
Terramycin	40	-	-	3	9	23	1	4

All of the *E. coli* strains were resistant to 5.0 units/ml of penicillin but were sensitive, with a few exceptions, to the four antibiotics. Streptomycin and chloromycetin appeared to be the most effective with average M.I.C's. of 7.0 and 8.4 mcg./ml respectively, whereas the corresponding figures for terramycin and aureomycin were 10.4 and 13.5 mcg./ml respectively.

Proteus.

In his report on the incidence of antibiotic resistance in Gram-negative organisms, Thomson (1952) found that 33 per cent of Pr. vulgaris strains were resistant to streptomycin, 88 per cent to aureomycin, 37 per cent to chloromycetin and 87 per cent to terramycin. The fact that Proteus is more sensitive to streptomycin and chloromycetin than to other antibiotics has been confirmed by a number of workers including Valentine and Shooter (1954), Frank, Wilcox and Finland (1950), Weil and Stempel (1953), MacFarlane (1951) and Poole (1954).

Three varieties of Proteus were recovered from infected canine ears and of these 72 mirabilis, 7 vulgaris and the single strain of Pr. morganii were tested for their sensitivities to five antibiotics.

Although each strain was examined by the disc diffusion method on horse blood agar plates irregular results were obtained as a result of the organisms characteristic spreading type of growth on this medium. Although swarming was prevented by using MacConkey's medium it appeared that the diffusion rates of the antibiotics were adversely affected by the presence of bile slats. In order to overcome these disadvantages the strains were classified, initially, as 'fully sensitive', 'moderately sensitive' or 'resistant', according to the results of the diffusion tests on blood agar, final results being determined by means of tube tests.

TABLE 136.

The in vitro sensitivities of Proteus to five antibiotics.

M.I.C. mcg/ml.	Penicillin			Streptomycin			Chloromycetin			Aureomycin			Terramycin		
	M. 72	V. 7	m. 1	M. 72	V. 7	m. 1	M. 72	V. 7	m. 1	M. 72	V. 7	m. 1	M. 72	V. 7	m. 1
>25.0	72	7	1	6	1	-	3	1	-	72	2	-	70	3	-
25.0	-	-	-	32	5	-	21	-	-	-	2	-	1	1	-
12.5	-	-	-	29	-	-	21	2	-	-	3	-	1	1	-
6.0	-	-	-	5	1	-	16	3	-	-	-	1	-	1	-
3.0	-	-	-	-	-	-	6	1	-	-	-	-	-	1	1
1.5	-	-	-	-	-	1	5	-	1	-	-	-	-	-	-
0.75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Average M.I.C.	RESISTANT			18.0	18.5	1.5	13.2	7.7	1.5	-	17.5	6.0	-	11.6	3.0

M.I.C. = Minimal Inhibiting Concentration in mcg./ml (Penicillin in units/ml.).

M. = Pr. mirabilis
V. = Pr. vulgaris
m. = Pr. morganii

Note: In the case of penicillin the highest concentration tested was 5 units /ml.

It will be seen from the results in Table 136 that the Proteus group as a whole, was resistant to penicillin at therapeutic levels, although an occasional strain was thought to be slightly sensitive at higher concentrations. Although the number of vulgaris and morganii strains was small, there appeared to be some correlation between the different species and their sensitivities to chloromycetin, streptomycin, aureomycin and terramycin. Most of the mirabilis and vulgaris strains were moderately sensitive to chloromycetin and streptomycin, the average M.I.C. for the mirabilis strains being 13.2 and 18.0 mcg./ml respectively and, for the vulgaris strains, 7.7 and 18.5 mcg./ml respectively. With the morganii strain, the M.I.C. of the same two antibiotics did not exceed 1.5 mcg./ml, whereas with terramycin and aureomycin values of 3.0 and 6.0 mcg./ml respectively, were obtained. Most of the vulgaris strains were sensitive to relatively higher concentrations of terramycin and aureomycin, in contrast to the mirabilis strains which were generally resistant.

Pseudomonas.

In addition to his findings with Proteus, to which reference has already been made, Thomson (1952) observed that 53.0, 92.8, 83.1 and 71.0 per cent of Ps. aeruginosa strains were resistant respectively, to the action of streptomycin, aureomycin, chloromycetin and terramycin. Valentine and Shooter (1954) considered the most effective antibiotics to be streptomycin,

aureomycin and terramycin, and chloromycetin in that order, while May and Morley (1952) found that 72 per cent were inhibited by 50.0 mcg./ml of terramycin, 2 per cent by a similar concentration of chloromycetin and 6 per cent by 25.0 mcg./ml of streptomycin.

The sensitivities to antibiotics of 85 strains of Ps. aeruginosa from infected canine ears was studied by disc diffusion methods, using either impregnated filter paper discs or proprietary "Sentests" (Evans Medical Supplies, Ltd.), the results in each case being checked by means of tube tests.

Table 137.

The in vitro sensitivities of 85 strains of Ps. aeruginosa to five antibiotics.

Antibiotic	Number of strains with the M.I.C. of :-								
	1.5-6.0	8.0	12.0	16.0	24.0	32.0	48.0	62.0	>62.0
Penicillin	-	-	-	-	-	-	-	-	85
Streptomycin	9	3	12	10	17	7	11	5	10
Chloromycetin	-	-	-	1	2	1	7	9	65
Aureomycin	-	-	-	-	3	4	23	35	24
Terramycin	2	-	1	5	14	19	21	9	14

Total no of strains examined = 85.

Although a few resistant aeruginosa strains were slightly sensitive to high concentrations of penicillin none was inhibited by less than 5 units/ml. The most effective antibiotics were Streptomycin and terramycin, with average M.I.C. of 24.3 and 36.7 mcg./ml respectively. Of the sensitive strains, 58 (77 per cent) were sensitive to 32.0 mcg./ml, or less, of streptomycin, whereas only 41 (58 per cent) were inhibited by the same amount of terramycin. Aureomycin and chloromycetin were less effective, as 24 (28 per cent) strains were resistant to the former and 65 (77 per cent) strains were resistant to the latter; the M.I.C. being 57.5 and 49.5 mcg./ml respectively.

TABLE 138.

Antibiotic sensitivity tests - summarised results.

Organism	No. of strains.	Penicillin (units/ml)	Streptomycin (mcg./ml.)	Chloromycetin (mcg./ml.)	Aureomycin (mcg./ml.)	Terramycin (mcg./ml.)
Staphylococci (canine)	180	0.02 - 0.3 R. (0.05)	0.1 - 25.0 R. (1.8)	0.75 - 12.5 R. (3.6)	0.05 - 3.0 (0.4)	0.1 - 3.0 (0.7)
Staphylococci (bovine)	45	0.02 - 2.0 R. (0.2)	0.1 - 12.5 (2.6)	0.75 - 25.0 R. (4.7)	0.05 - 8.0 (0.9)	0.75 - 3.0 (0.4)
Haemolytic streptococci	20	0.01 - 0.05 (0.02)	3.0 - 12.0 R. (6.5)	0.5 - 9.0 (3.4)	0.5 - 3.0 (1.2)	0.5 - 3.0 (1.4)
Non-haemolytic streptococci	20	0.2 - 1.0 (0.48)	12.0 - 30.0 R. (20.3)	6.0 - 30.0 (12.5)	3.0 - 12.0 (6.1)	3.0 - 20.0 (7.6)
E. coli	40	R R.	1.5 - 25.0 R. (7.0)	3.0 - 25.0 R. (8.4)	3.0 - 25.0 (13.5)	3.0 - 25.0 R. (10.4)
Pr. mirabilis	72	R R.	6.0 - 25.0 R. (18.0)	1.5 - 25.0R. (13.2)	R R.	R R +.
Pr. vulgaris	7	R R.	6.0 - 25.0 R. (18.5)	3.0 - 12.5 R. (7.7)	12.5 - 25.0 R. (17.5)	3.0 - 25.0 (11.6)
Pr. morganii	1	R R.	1.5	1.5	6.0	3.0
Ps. aeruginosa	85	R R.	1.5 - 25.0 R. (24.3)	16.0 - 62.0 R. (49.5)	24.0 - 62.0 R. (57.5)	1.5 - 62.0 R. (36.7)

Note: The numerals in brackets give the average M.I.C. for sensitive strains only.
R. = Resistant strains not uncommon.
R R. = Most strains resistant.
+. = Two strains were slightly sensitive to terramycin.

PART VII. - Summary.

Penicillin, terramycin and aureomycin were shown to be the most effective antibiotics, *in vitro*, against coagulase positive staphylococci of canine origin. Closely similar results were obtained with pathogenic bovine strains except that 60 per cent were resistant to 2 units/ml of penicillin compared with only 7 per cent of the canine strains.

The haemolytic streptococci were much more sensitive to antibiotics than were the non-haemolytic varieties and, apart from an occasional strain that resisted streptomycin, they were all sensitive to all five antibiotics. A possible correlation between the species of *Proteus* and their sensitivities to chloromycetin, streptomycin, aureomycin and terramycin was suggested by the fact that the *mirabilis* strains were sensitive *in vitro* to streptomycin and chloromycetin, while the *vulgaris* species were only moderately sensitive and the single strain of *Pr. morganii* was fully sensitive, to streptomycin, chloromycetin, aureomycin and terramycin.

The *E. coli* strains were resistant to penicillin but were sensitive to streptomycin, chloromycetin, terramycin and aureomycin, in that order.

Ps. aeruginosa was also resistant, at therapeutic levels, to penicillin and although some strains were fully sensitive to

to streptomycin and terramycin, the majority were only moderately sensitive to aureomycin and chloromycetin. It was also noticed that, although the average M.I.C. of streptomycin and terramycin was 24.3 and 36.7 mcg./ml respectively, a few strains were inhibited by concentrations of only 0.5 mcg./ml of either antibiotic. This is in agreement with the observations of Larner and Armstrong (1951) who noted a considerable degree of variation of sensitivities between, and within, organisms of the same species.

As a result of these investigations, the efficacy of the different antibiotic substances may be summarised as follows:-

Penicillin. very effective against staphylococci and streptococci but of little value, at therapeutic levels, against other bacteria.

Streptomycin. very effective against staphylococci and Pr. morganii; effective against E. coli and haemolytic streptococci but only moderately effective against other species. Nevertheless, it is probably the antibiotic of choice against Pseudomonas infections

Chloromycetin moderately effective against all but Pr morganii.

Aureomycin. very effective against staphylococci and streptococci, moderately effective against others, except Pseudomonas and Pr. mirabilis.

Terramycin. very effective or moderately effective against all but Pr. mirabilis; not quite so effective as streptomycin against Ps. aeruginosa.

Although it is not intended, in this thesis, to deal with the purely clinical aspects of canine otitis, the following points arise from the results of the in vitro antibiotic sensitivity tests.

The initial lesion of otitis, which was thought to be due to the extension of a skin condition or to local irritation by foreign bodies such as ear mites (Part IV), usually becomes infected with a variety of organisms which were classified as a) Gram-negative rods and b) Gram-positive cocci and yeasts. It is of interest that the Gram-negative species, Pseudomonas, Proteus and E. coli, which were usually associated with the purulent, chronic forms of otitis were, as a group, sensitive only to streptomycin, although the individual species were also sensitive to some of the other antibiotics. In the milder types of otitis the predominant organisms included staphylococci, almost all of which were fully sensitive to penicillin, streptomycin, aureomycin and terramycin and Pityrosporum species, many of which were sensitive to antiseptics.

These findings suggest that the treatment of canine external otitis should be directed firstly to the complete erradication of infection and secondly to the prompt healing of the ear lesion by treating it as a skin condition.

DISCUSSION

The importance of canine otitis is due not only to the very large number of dogs affected but also to the fact that many cases are extremely difficult to cure. Although the clinical aspects of the condition have been considered in some detail, the need for the present investigation was prompted by the fact that little is known of the nature of the causal agents of otitis or of the bacterial flora of the healthy canine ear.

As it is generally agreed that some breeds are more susceptible to otitis than others, it was interesting to find that the commonest of the 32 breeds examined were Spaniels and other long-haired dogs with overlapping ears. Although this type of ear favours the retention of moisture and debris, which may lead to maceration of the epithelium of the external acoustic meatus, many other factors may give rise to the primary lesion of otitis. Only in exceptional cases, however, did it appear that the lesion was due to injuries to the ear, nutritional deficiencies and the sequelae of debilitating and generalised infections. Of more importance was the fact that 13 per cent of the affected dogs were clinically infested with ectoparasites and, although ear mites did not appear to act as vectors or mechanical agents in the transmission of infection, it was evident that some of the milder, uncomplicated forms of otitis were due to irritation of the lining epithelium by Otodectes cyanotis. Fortunately such

such cases are of little practical importance as removal of the causal agents, whether they be ear mites, grit, grass awns or other irritants, is usually sufficient to effect a cure. The most important of the predisposing factors to otitis appeared to be the presence of skin diseases from which no fewer than 38 and 59 per cent respectively of the dogs in the unselected and chronic otitis groups were suffering. Not only was the extension to the external auditory meatus of a skin condition elsewhere on the animal's body thought to be responsible for the primary ear lesion, but the incidence of Proteus mirabilis and Pseudomonas aeruginosa was highest in such cases, whereas in dogs that were free from skin complaints the otitis was usually of the mild, non-purulent type in which only staphylococci and Pityrosporum species predominated.

The importance of bacteria and yeasts as the causal agents of otitis has received the attention of a number of workers both in the fields of veterinary and human medicine. Of the former, Farrag and Hosny Mahmoud (1953) and Gustafson (1954) claimed to have induced otitis by instilling saline suspensions of organisms into the ear canals of healthy dogs, although they failed to show conclusively that their results were not influenced by several physical factors which might have given rise to maceration of the meatal epithelium.

The fact that staphylococci and Pityrosporum were almost equally prevalent in healthy and infected ears suggested that the

the initial damage was due either to dermonecrotic or other staphylococcal toxins or to the irritant properties of Pityrosporum, akin to dandruff of the human scalp. It was shown, however, by treating a number of selected cases with suitable antibiotic and antiseptic preparations that the clinical condition did not improve appreciably even although the affected ears were apparently sterile on subsequent examinations.

On the other hand, examination of a number of purulent, chronic cases showed that in Proteus infections an antigenically identical strain to the ear strain was present in the intestines of the great majority of dogs, that the coliform organisms in the ears were mainly faecal type 1 of E. coli, and that several cases became infected with Pseudomonas, Proteus and coliforms only after the clinical condition was first observed.

The above findings suggest that extension of a skin condition to the external ear frequently gives rise to the primary lesion of otitis which, aggravated by scratching, probably becomes infected with commensal staphylococci or yeasts, or by contamination with faecal strains of Escherichia, Pseudomonas or Proteus from an external source or by auto-infection from the intestines. Other organisms which were frequently isolated from both healthy and affected external ears, and which were, therefore, thought to be of little importance, were aerobic sporing bacilli, diphtheroid bacilli, non haemolytic streptococci and anaerobic sporing bacilli most of which were Clostridium welchii. Haemolytic

Haemolytic streptococci were comparatively uncommon in otitic material, although most of the strains were of the potentially pathogenic Group G.

Although streptococci in Groups G. and M. were frequently present in the tonsils, otitis media did not appear to be a common condition in dogs. Of the few cases encountered, most were thought to be due to an external ear infection associated with Proteus or Pseudomonas with, or without, ulceration or perforation of the tympanum. That ascending infections by way of the eustachian tubes are also rare in dogs was suggested by the fact that Pasteurella species were isolated from less than one per cent of the infected ears, although they were present in the tonsils of 34 per cent of healthy dogs.

A point of clinical value arose from the fact that the bacterial findings were apparently related to the nature of the discharge in affected external ears. As staphylococci and Pityrosporum species were commonest in the dry, chocolate coloured exudates of the milder forms of otitis, whereas Proteus, Pseudomonas and coliform organisms were mostly present in the purulent discharges of the more chronic cases, it appeared possible that the nature of the discharge was related to the tissue responses to the different species of invading micro-organisms. In an attempt to confirm this, histological sections were prepared from infected external ears at three levels and of a number of clinically healthy ears. The only constant feature of the milder forms of otitis was thickening of the stratified

stratified squamous epithelium, whereas in most of the chronically infected ears the thickened epithelium showed marked rete-peg formation, ulceration and a degree of fibrosis that frequently resulted in constriction of the lumen of the external ear canal. Of more importance, in these latter cases, were the changes which occurred in the glandular tissues, the sebaceous follicles being mostly displaced in the superficial dermal layers by numerous, cystic diverticula of the modified ceruminous, or apocrine, glands. These enormously large, glandular structures which, in healthy ears, are very small and are usually to be seen only in the deeper layers of the corium, were frequently distended with an homogeneous, eosinophilic, colloidal secretion due, it was thought, not only to the increased activity of the tubular glands in infected ears but also to partial blockage of their excretory ducts by inflammatory changes in the superficial epithelium. Although only a limited number of ears were examined histologically, the tissue changes appeared to be more closely related to the type of discharge and the identity of the infectious agent than to the duration of the condition.

Although detailed examination of the more frequently occurring micro-organisms in canine external otitis showed them to be biochemically similar to strains from human and certain other animal sources, the staphylococci were exceptional in that several important characters were peculiar to strains of canine origin.

In the early stages of this work, the true nature of the dog

dog staphylococci was obscured by the fact that they usually failed to produce pigment on milk agar media and rarely coagulated human plasma. That these findings were confirmed by a study of a number of known pathogenic strains from other staphylococcal infections in dogs, suggested that their staphylocoagulase was different from that of other animal staphylococci. It was also found that 85 per cent of the dog strains coagulated rabbit plasma compared with only 11 per cent with human plasma, but that over 90 per cent of the pathogenic staphylococci from other animal sources promptly coagulated both rabbit and human plasmas. Although 72 per cent of the other animal staphylococci formed alpha haemolysin, the presence of which is held to be a reliable indication of pathogenicity, only 34 per cent of the dog strains formed true alpha toxin or a new haemolysin which it closely resembled. Moreover, with dog staphylococci, coagulase activity was more closely related to the formation of delta haemolysin, which was produced by 93 per cent of the coagulase positive strains but only by 2 per cent of the coagulase negative strains. Other characters which distinguished the dog staphylococci from those from other animal sources were the prompt liquefaction of gelatin, the hydrolysis of casein and inspissated bovine serum, the delayed fermentation of mannitol and the inability to grow well in the presence of bile salts.

Apart from Pityrosporum, other yeasts and fungi did not appear to play an important part in canine otitis, although a

a few cases of otomycosis due to Candida tropicalis were encountered in the earlier part of this work when the routine method of treatment included the use of penicillin.

The in vitro sensitivities to antibiotics of a number of the more frequently occurring bacteria in infected ears showed that there was no evidence, as yet, of acquired resistance to antibiotics by staphylococci and other bacteria of canine origin.

The results of this work might well suggest that a rational method of treatment of external canine otitis would be, firstly, to remove the predominant bacteria in infected ears by the use of antibiotics, either singly or in combination, and Pityrosporum and other yeasts by suitable fungicides, with steps being taken to promote rapid healing of the ear lesion before re-infection can occur.

SUMMARY

The commonest breeds to be affected with otitis were Spaniels and other long-haired dogs with overlapping ears.

The most important of the predisposing factors to otitis were skin diseases from which 38 and 59 per cent respectively of the dogs in the unselected and chronic otitis groups were suffering. Only 13 per cent of the dogs were infested with ectoparasites.

In most cases the primary lesion of otitis was due to the extension of a skin condition to the external acoustic meatus or, occasionally, to irritation by ear mites and foreign bodies such as grit and grass awns.

Infection of the ear lesion was due either to commensal staphylococci or yeasts, or to faecal contamination with Pseudomonas, Proteus or coliform organisms.

The incidence of Pseudomonas and Proteus was greatest in dogs with chronic otitis that were also suffering from a clinical skin disease. In a number of cases infection occurred during, or shortly after, a course of treatment with antibiotics.

Pseudomonas, Proteus and coliform infections were generally associated with the copious, purulent types of discharge, whereas staphylococci and Pityrosporum predominated in the dryish, dark-coloured exudates.

The tissue changes in affected ears were more closely related

related to the nature of the discharge and the identity of the infectious agent than to the duration of the condition. The numerous, large, cystic diverticula of the tubular portions of the modified ceruminous, or apocrine, glands were a constant feature of the more purulent forms of otitis.

All the Pseudomonas strains from infected ears were identified as Ps. aeruginosa, a number of which were sensitive to phages of human strains.

Pr. mirabilis accounted for 95 per cent of the Proteus strains, about half of which were antigenically similar. To some extent, species identification was determined by the strain's in vitro sensitivities to antibiotics.

The commonest coliform organisms recovered from infected ears were faecal type 1 of E. coli.

Unlike Group G. haemolytic streptococci, Group M. strains were rarely isolated from otitic material, although they were frequently present in the tonsils of healthy dogs.

Canine staphylococci differed markedly from those from human and certain other animal sources. Most of the dog strains were non-pigmented and coagulated rabbit but not human plasmas. Their coagulase activity was positively correlated with the production of delta but not alpha haemolysin, while a number of coagulase positive strains formed a new haemolysin. Of the few (7 per cent) penicillin resistant dog staphylococci, most were of the alpha-delta haemolytic pattern and produced aureus pigment which

which are features of human, rather than canine, strains.

Pityrosporum species were isolated from most of the healthy and infected external ears, but from no other site, and differed from other members of the genus by growing well in maltose agar without the addition of oleic acid.

ACKNOWLEDGEMENTS

The writer wishes to express his thanks to the late Professor T.J. Mackie for guidance in the early stages of this work, to Dr. A. Wilson Taylor for his help and encouragement and to Dr. R.H.A. Swain for much valuable criticism.

He is very grateful to Dr. J.C. Gould for phage typing the Pseudomonas strains, to Mr. A.R. Withers, Mr. W.W. Gregor and the staff of the Small Animal Clinic of this School for their help in obtaining material, to Mr. R.C.W.S. Hood for the photography and to Messrs. Burroughs Wellcome for antisera.

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APPENDIX 1.

The flora of the external ears (at three levels), the middle ears, the nares and the tonsils of clinically healthy dogs.

Case No.		External ears: Pinna. A	B	C	D	E	F	G	H	Distal to angle of meatus A	B	C	D	E	F	G	H	Proximal to angle of meatus A	B	C	D	E	F	G	H	Middle ears A	B	C	D	E	F	G	H	Anterior nares A	B	C	D	E	F	G	H	Tonsils A	B	C	D	E	F	G	H
1	L.	+		+	+	+	+	+		+			+		+	+			+				+				+								+	+	+	+		+	+	+		+	+	+	+	+		+	+
	R.	+	+		+		+	+		+	+				+							+	+					+																						
2	L.	+			+	+		+	+				+		+	+	+						+									+				+	+	+	+	+		+		+	+	+		+	+
	R.	+			+			+	+	+			+	+	+	+					+																												
3	L.	+			+	+	+	+	+	+					+				+				+									+	+			+					+	+	+	+	+			+
	R.	+			+		+	+	+	+									+								+																						
4	L.	+	+		+	+	+	+		+			+			+	+		+				+									+	+			+					+	+	+		+			+
	R.				+	+		+	+				+	+			+		+				+																										
5	L.	+			+			+		+			+						+				+									+		+	+	+	+		+			+	+	+	+	+	+		+
	R.				+			+					+						+																														
6	L.	+							+																										+	+	+	+			+			+	+	+			+
	R.								+		+					+				+																												
7	L.	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+		+		+	+	+				+	+				+	+			+	+	+	+	+		+	+			+	+	+	+
	R.	+			+		+	+		+	+			+	+	+			+																													
8	L.				+		+	+	+				+			+	+				+				+										+			+				+							+
	R.	+	+		+	+		+	+	+			+	+	+	+	+		+				+																										
9	L.	+					+	+		+					+												+									+	+	+			+					+	+		+
	R.	+			+			+		+						+											+																					
10	L.	+	+	+	+	+	+	+	+	+	+		+		+	+	+		+			+	+				+	+				+				+	+	+			+	+			+	+	+	+
	R.	+			+	+	+	+	+	+			+	+			+		+		+																												

Legend: A = staphylococci B = streptococci C = Gram-negative rods D = aerobic sporing bacilli
E = diptheroids F = yeasts & fungi G = anaerobic sporing bacilli H = other bacteria.

L = left ear R = right ear

APPENDIX 2.

The aerobic micro-organisms in 70 clinically healthy external ears.

The coliform group.

1 Coliforms.
1 " + N.H. strept.
1 " + " + staph.
1 " + " + " + pos B.

4 ears (3 dogs) infected with coliforms.

Haemolytic streptococci.

2 Hy. strept. + staph. + pos B.

2 ears (2 dogs) infected with haemolytic streptococci.

Non-haemolytic streptococci.

1 NH. strept.
7 " + staph.
2 " + " + dipth.
2 " + " + pos B. + other species.
2 " + " + other species.
2 " + other species + pos B.
2 " + other species.

1 " + coliforms.
1 " + " + pos B. + staph. + other species.
1 " + " + staph. + other species.

21 ears (17 dogs) infected with non-haemolytic streptococci.

Healthy ears (contd.).

Staphylococci.

10 Staph.
3 " + pos. B.
1 " + " + dipth.
2 " + " + " + other species.
2 " + " + other species.
1 " + dipth. + other species.
2 " + other species.

1 " + coliforms + NH. strept. + other species.
1 " + " + " + " + pos B.
2 " + Hy. strept. + pos B.
7 " + NH. strept.
2 " + " + dipth.
2 " + " + pos B. + other species.
2 " + " + other species.

38 ears (27 dogs) infected with staphylococci.

Diptheroid bacilli.

2 Dipth.
1 " + pos B.
1 " + " + other species.
1 " + other species.

2 " + NH. strept. + staph.
1 " + staph. + pos B.
2 " + " + " + other species.
1 " + " + other species.

11 ears (10 dogs) infected with diptheroid bacilli.

Healthy ears (contd.).

Aerobic Gram-positive sporing bacilli.

2 Pos. B.
3 " + other species.

.....

2 " + Hy. strept. + staph.
2 " + NH. strept. + other species.
2 " + " + staph. + other species.
1 " + staph. + dipth.
2 " + " + other species.
3 " + staph.
2 " + " + dipth. + other species.
1 " + dipth. + other species.
1 " + dipth.
1 " + coliforms + NH. strept. + staph. + other species

---

22 ears (15 dogs) infected with aerobic sporing bacilli.

Sterile cultures.

Cultures prepared from 11 ears (11 dogs) were apparently sterile.

Legend:

Staph. = staphylococci
Strept. = streptococci
Dipth. = diptheroid bacilli
Pos B. = aerobic sporing bacilli.

Hy. = beta haemolytic
aHy. = alpha haemolytic
NH. = non haemolytic

APPENDIX 3.

The aerobic micro-organisms in the external ears of dogs suffering from otitis.

Pseudomonas.

23	Pseudomonas.	
10	"	+ Proteus.
4	"	+ " + coliforms + Hy. strept. + staph.
1	"	+ " + Hy. strept. + staph.+ dipth +pos B.
1	"	+ " + " + dipth.
1	"	+ " + staph. + dipth.
1	"	+ " + coliforms + NH. strept.
2	"	+ " + NH. strept.
1	"	+ coliforms + dipth. + pos B.
1	"	+ " + Hy. strept.
2	"	+ " + " + staph. + dipth.
1	"	+ " + " + dipth.
1	"	+ " + NH. strept.
1	"	+ " + " + staph. + dipth.
3	"	+ " + staph.
1	"	+ " + " + dipth.
1	"	+ " + pos B.
2	"	+ Hy. strept. + dipth.
1	"	+ " + staph.
2	"	+ " + pos B.
1	"	+ aHy. strept.
1	"	+ " + staph.
2	"	+ staph. + NH. strept.
1	"	+ " + dipth.
2	"	+ dipth. + pos B.

67 ears (51 dogs) infected with Pseudomonas.

Otitic material (contd.).

Proteus.

20 Proteus.
1 " + coliforms + aHy. strept.
1 " + " + Hy. strept. + staph.
2 " + " + NH. strept. + " + pos B.
1 " + " + " + " + dipth.
2 " + " + staph.
1 " + " + " + dipth.
1 " + Hy. strept.
2 " + " + pos B.
3 " + " + dipth.
1 " + " + NH. strept. + pos B.
1 " + " + " + staph. + dipth.
1 " + aHy. strept
1 " + " + staph.
1 " + NH. strept.
1 " + " + dipth.
4 " + " + staph.
2 " + " + " + dipth.
1 " + " + " + " + pos B.
1 " + " + pos B.
9 " + staph.
3 " + " + dipth.
1 " + " + " + pos B.
1 " + dipth.

.....

10 " + Pseudomonas.
4 " + " + coliforms + Hy. strept. + staph.
1 " + " + " + NH. strept.
1 " + " + Hy. strept. + dipth.
1 " + " + " + staph. + dipth. + pos B
1 " + " + staph. + dipth½
2 " + " + NH. strept.

---

82 ears (61 dogs) infected with Proteus. - 77 Pr. mirabilis.
4 Pr. vulgaris.
1 Pr. morganii.

Otitic material (contd.).

Coliform organisms.

7	Coliforms.					
1	"	+ Hy. strept.				
1	"	+ "	+ aHy. strept.			
2	"	+ "	+ "	+ staph.		
2	"	+ "	+ NH. strept.	+ staph.		
1	"	+ "	+ staph.			
1	"	+ "	+ "	+ dipth.		
2	"	+ "	+ "	+ "	+ pos B.	
1	"	+ "	+ "	+ pos B.		
1	"	+ aHy. strept.				
2	"	+ NH. strept.				
9	"	+ "	+ staph.			
2	"	+ "	+ "	+ dipth.		
1	"	+ "	+ dipth.			
8	"	+ staph.				
2	"	+ "	+ dipth.			
2	"	+ "	+ "	+ pos B.		
3	"	+ "	+ pos B.			
.....						
4	"	+ Pseudomonas	+ Proteus	+ Hy. strept.	+ staph.	
1	"	+ "	+ "	+ NH. strept.		
1	"	+ "	+ HY. strept.			
2	"	+ "	+ "	+ staph.	+ dipth.	
1	"	+ "	+ "	+ dipth.		
1	"	+ "	+ NH. strept.			
1	"	+ "	+ "	+ staph.	+ dipth.	
3	"	+ "	+ staph.			
1	"	+ "	+ "	+ dipth.		
1	"	+ "	+ dipth.	+ pos B.		
1	"	+ "	+ pos B.			
1	"	+ Proteus.	+ aHy. strept.			
1	"	+ "	+ Hy. strept.	+ staph.		
2	"	+ "	+ NH. strept.	+ staph.	+ pos B.	
1	"	+ "	+ "	+ "	+ dipth.	
2	"	+ "	+ staph.			
1	"	+ "	+ "	+ dipth.		

---

73 ears, (60) dogs, infected with coliform organisms.

Otitic material (contd.).

Beta haemolytic streptococci.

8 Hy. strept.
1 " + aHy. strept.
1 " + NH. strept.
4 " + " + staph.
1 " + " + " + dipth.
31 " + staph.
1 " + " + aHy. strept.
6 " + " + dipth.
4 " + " + " + pos B.
1 " " " + " + Pasteurella.
1 " + dipth.
1 " + pos B.

.....

4 " + Pseudo. + Proteus + coliforms + staph.
1 " + " + " + staph. + dipth. + pos B.
1 " + " + " + dipth.
1 " + " + coliforms.
2 " + " + " + staph. + dipth.
1 " + " + " + dipth.
1 " + " + staph.
2 " + " + dipth.
2 " + " + pos B.
1 " + Proteus.
1 " + " + coliforms + staph.
1 " + " + NH. strept. + staph. + dipth.
1 " + " + " + pos B.
3 " + " + dipth.
2 " + " + pos B.
1 " + coliforms.
1 " + " + aHy. strept.
2 " + " + " + staph.
2 " + " + NH. strept.+ staph.
1 " + " + staph.
1 " + " + " + dipth.
2 " + " + " + " + pos B.
1 " + " + " + pos B.

---

95 ears (78 dogs) infected with beta haemolytic streptococci.

Otitic material (contd.).

Alpha haemolytic streptococci.

10 aHY. strept.
1 " + NH. strept. + staph. + pos B.
1 " + " + pos B.
9 " + staph.
1 " + pos B.

.....

1 " + Pseudomonas + staph.
1 " + Pseudomonas
1 " + Proteus.
1 " + " + coliforms.
1 " + " + staph.
1 " + coliforms.
1 " + " + Hy. strept.
2 " + " + " + staph.
1 " + staph. + Hy. strept.
1 " + Hy. strept.

---

33 ears (28 dogs) infected with alpha haemolytic strept.

Otitic material (contd.).

Non-haemolytic streptococci.

5	NH. strept.	
40	"	+ staph.
6	"	+ " + dipth.
1	"	+ " + " + pos B.
2	"	+ " + pos B.

.....

1	"	+ Pseudomonas + Proteus + coliforms.
2	"	+ " + Proteus.
1	"	+ " + coliforms.
1	"	+ " + " + staph. + dipth.
2	"	+ " + staph.
1	"	+ Proteus + coliforms + staph. + dipth.
2	"	+ " + " + " + pos B.
1	"	+ " + Hy. strept+ staph. + dipth.
1	"	+ " + " + pos B.
1	"	+ " + dipth.
4	"	+ " + staph.
2	"	+ " + " + dipth.
1	"	+ " + " + " + pos B.
1	"	+ " + pos B.
1	"	+ Proteus.
2	"	+ coliforms + Hy. strept. + staph.
2	"	+ coliforms.
9	"	+ " + staph.
2	"	+ " + " + dipth.
1	"	+ " + dipth.
1	"	+ Hy. strept.
4	"	+ " + staph.
1	"	+ " + " + dipth.
1	"	+ aHY. strept. + staph. + pos B.
1	"	+ " + pos B.

---

100 ears (85 dogs) infected with non-haemolytic strept.

Otitic material (contd.).

Staphylococci.

101 Staph.
15 " + dipth.
5 " + " + pos B.
11 " + pos B.

.....

4 " + Pseudomonas + Proteus + coliforms + Hy strept.
1 " + " + " + Hy strept + dipth + pos B.
1 " + " + " + dipth.
2 " + " + coliforms + Hy. strept. + dipth
1 " + " + " + NH. strept. + dipth
3 " + " + coliforms
1 " + " + " + dipth.
1 " + " + Hy. strept.
1 " + " + aHy. strept.
2 " + " + NH. strept.
1 " + " + dipth.
1 " + Proteus + coliforms + Hy. strept.
2 " + " + " + NH. strept. + pos B.
1 " + " + " + " + dipth.
2 " + " + coliforms.
1 " + " + " + dipth.
1 " + " + Hy. strept + NH. strept+ dipth.
1 " + " + aHy. strept.
4 " + " + NH. strept.
2 " + " + " + dipth.
1 " + " + " + " + pos B.
3 " + " + dipth.
1 " + " + " + pos B.
9 " + Proteus.

continued over:

Otitic material (contd.).

Staphylococci (contd).

2	Staph.	+	coliforms	+	Hy. strept.	+	aHy. strept.	
2	"	+	"	+	"	+	NH. strept.	
1	"	+	"	+	Hy. strept.			
1	"	+	"	+	"	+	dipth.	
2	"	+	"	+	"	+	"	+ pos B.
1	"	+	"	+	"	+	pos B.	
9	"	+	"	+	NH. strept.			
2	"	+	"	+	"	+	dipth.	
8	"	+	coliforms.					
2	"	+	"	+	dipth.			
2	"	+	"	+	"	+	pos B.	
3	"	+	"	+	pos B.			
31	"	+	Hy. strept.					
4	"	+	"	+	NH. strept.			
1	"	+	"	+	"	+	dipth.	
1	"	+	"	+	aHy. strept.			
6	"	+	"	+	dipth.			
4	"	+	"	+	"	+	pos B.	
1	"	+	"	+	"	+	Pasteurella.	
1	"	+	aHy. strept.	+	NH. strept.	+	pos B.	
9	"	+	aHy. strept.					
40	"	+	NH. strept.					
6	"	+	"	+	dipth.			
1	"	+	"	+	"	+	pos B.	
2	"	+	"	+	dipth.			

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321 ears (251 dogs) infected with staphylococci.

Otitic material (contd).

Diptheroid bacilli.

8 Dipth.
2 " + pos B.

1 " + Pseudomonas.
1 " + " + Proteus + Hy. strept.
1 " + " + " + " + staph + pos B.
1 " + " + " + staph.
1 " + " + coliforms + pos B.
1 " + " + " + Hy. strept.
2 " + " + " + " + staph.
1 " + " + " + NH. strept + staph.
1 " + " + " + staph.
2 " + " + Hy. strept.
1 " + " + staph.
2 " + " + pos B.
1 " + Proteus. + coliforms + staph.
1 " + " + " + " + NH. strept.
3 " + " + Hy. strept.
1 " + " + " + NH. strept. + staph.
1 " + " + NH. strept.
2 " + " + " + staph.
1 " + " + " + " + pos B.
3 " + " + staph.
1 " + " + " + pos B.
1 " + coliforms. + Hy. strept. + staph.
2 " + " + " + " + pos B.
1 " + " + NH. strept.
2 " + " + " + staph.
2 " + " + staph.
2 " + " + " + pos B.
1 " + Hy. strept.
1 " + " + NH. strept. + staph.
6 " + " + staph.
4 " + " + " + pos B.
1 " + " + " + Pasteurella.
6 " + NH. strept + staph.
1 " + " + " + pos B.
15 " + staph.
5 " + " + pos B.

89 ears infected with diptheroid bacilli.

Otitic material (contd).

Aerobic sporing bacilli.

10 Pos B.

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1	Pos B.	+ Pseudo.	+ Proteus	+ Hy. strept	+ staph	+ dipth.
1	"	+ "	+ coliforms.			
1	"	+ "	+ "	+ dipth.		
2	"	+ "	+ Hy. strept.			
2	"	+ "	+ dipth.			
2	"	+ Proteus	+ coliforms.	+ NH. strept.	+ staph.	
2	"	+ "	+ Hy. strept.			
1	"	+ "	+ "	+ NH. strept.		
1	"	+ "	+ NH. strept.			
1	"	+ "	+ "	+ staph.	+ dipth.	
1	"	+ "	+ staph.	+ dipth.		
2	"	+ coliforms.	+ Hy. strept.	+ staph.	+ dipth.	
1	"	+ "	+ "	+ staph.		
2	"	+ "	+ staph.	+ dipth.		
3	"	+ "	+ staph.			
1	"	+ Hy. strept.				
4	"	+ "	+ staph.	+ dipth.		
1	"	+ aHy. strept.				
1	"	+ "	+ NH. strept.			
1	"	+ "	+ "	+ staph.		
2	"	+ NH. strept.	+ staph.			
1	"	+ "	+ "	+ dipth.		
11	"	+ staph.				
5	"	+ "	+ dipth.			
2	"	+ dipth.				

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62 ears infected with aerobic sporing bacilli.